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PATENT
454312-2400

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION FOR LETTERS PATENT

Title: PNEUMOCOCCAL GENES, PORTIONS THEREOF, EXPRESSION PRODUCTS THEREFROM, AND USES OF SUCH GENES, PORTIONS AND PRODUCTS

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PATENT
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PNEUMOCOCCAL GENES, PORTIONS THEREOF, EXPRESSION PRODUCTS THEREFROM, AND USES OF SUCH GENES, PORTIONS AND PRODUCTS

RELATED APPLICATIONS

This application is a continuation-in-part ("CIP"): of application Serial Nos. 08/226,844 08/093,907 07/884,916, filed May 29, 1992, July 5, 1994 (corresponding to PCT/US93/05191); of application Serial No. 08/482,981, filed June 7, 1995; of application Serial No. 08/458,399, filed June 2, 1995; of application Serial No. 08/446,201, filed May 19, 1995 (as a CIP of USSN 08/246,636); of application Serial No. 08/246,636, filed May 20, 1994 (as a CIP of USSN 08/048,896, filed April 20, 1993 as a CIP of USSN 07/835,698, filed February 12, 1992 as a CIP of USSN 07/656,773); of application Serial 08/319,795, filed October 7, 1994 (as a CIP of USSN 08/246,636); of application Serial No. 08/072,070, filed June 3, 1993; of application Serial No. 07/656,773, filed February 15, 1991 (USSN 656,773 and 835,698 corresponding to Int'l application WO 92/1448); and, each of these applications, as well as each application, document or reference cited in these applications, is hereby incorporated herein by reference. Documents or references are also cited in the following text, either in a Reference List appended to certain Examples, or before the claims, or in the text itself; and, each of these documents or references is hereby expressly incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to pneumococcal genes, portions thereof, expression products therefrom and uses of such genes, portions and products; especially to genes of *Streptococcus pneumoniae*, e.g., the gene encoding pneumococcal surface protein A (PspA) (said gene being "*pspA*"), *pspA*-like genes, portions of such genes, expression products therefrom, and the uses of such genes, portions thereof and expression products therefrom. Such uses include uses of the genes and portions thereof for obtaining expression products by recombinant techniques, as well as for detecting the presence of *Streptococcus pneumoniae* or strains thereof by detecting DNA thereof by hybridization or amplification (e.g., PCR) and hybridization techniques (e.g., obtaining DNA-containing sample, contacting same with genes or fragment under PCR, amplification and/or hybridization conditions, and detecting presence of or isolating hybrid or amplified product). The expression product uses include use in preparing antigenic, immunological or vaccine compositions, for eliciting antibodies, an immunological response (other than or additional to antibodies) or a protective response (including antibody or other immunological response by administering composition to a suitable host); or, the expression product can be for use in detecting the presence of *Streptococcus pneumoniae* by detecting antibodies to *Streptococcus pneumoniae* protein(s) or antibodies to a portion thereof in a host, e.g., by obtaining an

antibody-containing sample from a relevant host, contacting the sample with expression product and detecting binding (for instance by having the product labeled); and, the antibodies generated by the aforementioned compositions are useful in diagnostic or detection kits or assays. Thus, the invention relates to varied compositions of matter and methods for use thereof.

BACKGROUND OF THE INVENTION

Streptococcus pneumoniae is an important cause of otitis media, meningitis, bacteremia and pneumonia. Despite the use of antibiotics and vaccines, the prevalence of pneumococcal infections has declined little over the last twenty-five years.

It is generally accepted that immunity to *Streptococcus pneumoniae* can be mediated by specific antibodies against the polysaccharide capsule of the pneumococcus. However, neonates and young children fail to make an immune response against polysaccharide antigens and can have repeated infections involving the same capsular serotype.

One approach to immunizing infants against a number of encapsulated bacteria is to conjugate the capsular polysaccharide antigens to protein to make them immunogenic. This approach has been successful, for example, with *Haemophilus influenzae b* (see U.S. Patent no. 4,496,538 to Gordon and U.S. Patent no. 4,673,574 to Anderson). However, there are over eighty known capsular serotypes of *S. pneumoniae* of which twenty-three account for most

of the disease. For a pneumococcal polysaccharide-protein conjugate to be successful, the capsular types responsible for most pneumococcal infections would have to be made adequately immunogenic. This approach may be difficult, because the twenty-three polysaccharides included in the presently-available vaccine are not all adequately immunogenic, even in adults.

An alternative approach for protecting children, and also the elderly, from pneumococcal infection would be to identify protein antigens that could elicit protective immune responses. Such proteins may serve as a vaccine by themselves, may be used in conjunction with successful polysaccharide-protein conjugates, or as carriers for polysaccharides.

McDaniel et al. (I), J. Exp. Med. 160:386-397, 1984, relates to the production of hybridoma antibodies that recognize cell surface polypeptide(s) on *S. pneumoniae* and protection of mice from infection with certain strains of encapsulated pneumococci by such antibodies. This surface protein antigen has been termed "pneumococcal surface protein A" or PspA for short.

McDaniel et al. (II), Microbial Pathogenesis 1:519-531, 1986, relates to studies on the characterization of the PspA. Considerable diversity in the PspA molecule in different strains was found, as were differences in the epitopes recognized by different antibodies.

McDaniel et al. (III), J. Exp. Med. 165:381-394, 1987, relates to immunization of X-linked immunodeficient (XID) mice

with non-encapsulated pneumococci expressing PspA, but not isogenic pneumococci lacking PspA, protects mice from subsequent fatal infection with pneumococci.

McDaniel et al. (IV), Infect. Immun., 59:222-228, 1991, relates to immunization of mice with a recombinant full length fragment of PspA that is able to elicit protection against pneumococcal strains of capsular types 6A and 3.

Crain et al, Infect.Immun., 56:3293-3299, 1990, relates to a rabbit antiserum that detects PspA in 100% (n = 95) of clinical and laboratory isolates of strains of *S. pneumoniae*. When reacted with seven monoclonal antibodies to PspA, fifty-seven *S. pneumoniae* isolates exhibited thirty-one different patterns of reactivity.

The PspA protein type is independent of capsular type. It would seem that genetic mutation or exchange in the environment has allowed for the development of a large pool of strains which are highly diverse with respect to capsule, PspA, and possibly other molecules with variable structures. Variability of PspA's from different strains also is evident in their molecular weights, which range from 67 to 99 kD. The observed differences are stably inherited and are not the result of protein degradation.

Immunization with a partially purified PspA from a recombinant λ gt11 clone, elicited protection against challenge with several *S. pneumoniae* strains representing different

capsular and PspA types, as described in McDaniel et al. (IV), Infect. Immun. 59:222-228, 1991. Although clones expressing PspA were constructed according to that paper, the product was insoluble and isolation from cell fragments following lysis was not possible.

While the protein is variable in structure between different pneumococcal strains, numerous cross-reactions exist between all PspA's, suggesting that sufficient common epitopes may be present to allow a single PspA or at least a small number of PspA's to elicit protection against a large number of *S. pneumoniae* strains.

In addition to the published literature specifically referred to above, the inventors, in conjunction with co-workers, have published further details concerning PspA's, as follows:

1. Abstracts of 89th Annual Meeting of the American Society for Microbiology, p. 125, item D-257, May 1989;

2. Abstracts of 90th Annual Meeting of the American Society for Microbiology, p. 98, item D-106, May 1990;

3. Abstracts of 3rd International ASM Conference on Streptococcal Genetics, p. 11, item 12, June 1990;

4. Talkington et al, Infect. Immun. 59:1285-1289, 1991;

5. Yother et al (I), J. Bacteriol. 174:601-609, 1992; and

6. Yother et al (II), J. Bacteriol. 174:610-618, 1992.

7. McDaniel et al (V), Microbiol. Pathogenesis, 13:261-268.

It would be useful to provide PspA or fragments thereof in compositions, including PspA's or fragments from varying strains in such compositions, to provide antigenic, immunological or vaccine compositions; and, it is even further useful to show that the various strains can be grouped or typed, thereby providing a basis for cross-reactivities of PspA's or fragments thereof, and thus providing a means for determining which strains to represent in such compositions (as well as how to test for, detect or diagnose one strain from another). Further, it would be advantageous to provide evidence of a *pspA* - like gene or second *pspA* gene in certain strains, as well as primers (oligonucleotides) for identification of such a gene, as well as of conserved regions in that gene and in *pspA*; for instance, for detecting, determining, isolating, or diagnosing strains of *S. pneumonia*. These uses and advantages, it is believed, have not heretofore been provided in the art.

SUMMARY OF THE INVENTION

The invention provides an isolated amino acid molecule comprising of residues 1 to 115, 1 to 260, 192 to 588, 192 to 299, or residues 192 to 260 of pneumococcal surface protein A of *Streptococcus pneumoniae*.

The invention further provides an isolated DNA molecule comprising of a fragment of a pneumococcal surface protein A gene of *Streptococcus pneumoniae* encoding the isolated amino acid molecule.

The invention also provides PCR primer or hybridization probe comprising the isolated DNA molecule.

The invention additionally provides an antigenic, vaccine or immunological composition comprising the amino acid molecule.

The invention includes an isolated DNA molecule comprising nucleotides 1 to 26, 1967 to 1990, 161 to 187, 1093 to 1117, or 1312 to 1331 or 1333 to 1355 of a pneumococcal surface protein A gene of *Streptococcus pneumoniae*. The DNA molecule can be used as a PCR primer or hybridization probe; and therefore the invention comprehends a PCR primer or hybridization probe comprising of the isolated DNA molecule.

The invention also includes an isolated DNA molecule comprising (a) fragment (having homology with a portion) of a pneumococcal surface protein A gene of *Streptococcus pneumoniae* (the DNA preferably is) comprising a nucleotide sequence (5′ to 3′) selected from the following (which include the portion having homology and restriction sites) [selection of other restriction sites or sequences for such DNA is within the ambit of the skilled artisan from this disclosure]:

CCGGATCCAGCTCCTGCACCAAAAAC;
GCGCGTCGACGGCTTAAACCCATTCACCATTGG;
CCGGATCCTGAGCCAGAGCAGTTGGCTG;
CCGGATCCGCTCAAAGAGATTGATGAGTCTG;
GCGGATCCCGTAGCCAGTCAGTCTAAAGCTG;
CTGAGTCGACTGGAGTTTCTGGAGCTGGAGC;
CCGGATCCAGCTCCAGCTCCAGAAACTCCAG;
GCGGATCCTTGACCAATATTTACGGAGGAGGC;
GTTTTTGGTGCAGGAGCTGG;
GCTATGGCTACAGGTTG;
CCACCTGTAGCCATAGC;
CCGGATCCAGCGTGCCTATCTTAGGGGCTGGTT; and
GCAAGCTTATGATATAGAAATTTGTAAC.

(Thus, the invention broadly comprehends DNA homologous to portions of pspA; preferably further including restriction sequences).

These DNA molecules can be used as PCR primers or probes; and thus, the invention comprehends a primer or probe comprising any of these molecules.

The invention further still provides PCR probe(s) which distinguishes between *pspA* and *pspA*-like nucleotide sequences, as well as PCR probe(s) which hybridizes to both *pspA* and *pspA*-like nucleotide sequences.

Additionally, the invention includes a PspA extract prepared by a process comprising: growing pneumococci in a first medium containing choline chloride, eluting live pneumococci with a choline chloride containing salt solution, and growing the pneumococci in a second medium containing an alkanolamine and substantially no choline; as well as a PspA extract prepared by that process and further comprising purifying PspA by isolation on a choline-Sepharose affinity column. These processes are also included in the invention.

An immunological composition comprising these extracts is comprehended by the invention, as well as an immunological composition comprising full length PspA.

A method for enhancing immunogenicity of a PspA-containing immunological composition comprising including in said composition the C-terminal portion of PspA, is additionally comprehended, as well as

An immunological composition comprising at least two PspAs. The latter immunological composition can have the PspAs from different groups or families; the groups or facsimile can be based on RFLP or sequence studies (see, e.g., Fig. 13).

These and other embodiments are disclosed or are obvious from the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B show: Evaluation of digested plasmid constructs. Fig. 1A: 1% agarose gel electrophoresis of plasmids isolated from transformed *E. coli* BL21(DE3) strains stained with ethidium bromide. Lane 1: 1 kb DNA ladder (sizes noted in kb), lane 2: pRCT125; lane 3: pRC105, lane 4: DBL5 pspA insert, lane 5: pRCT113, lane 6: BG9739 pspA insert, lane 7: pRCT117, and lane 8: L81905 pspA insert. Fig. 1B: Corresponding Southern blot of gel in Fig. 1A probed with full-length Rx1 pspA and hybridization detected as described in Example 1. The arrow indicates the 1.2 kb pspA digested inserts from plasmid constructs and the PCR-amplified pspA fragments from the pneumococcal donor strains used in cloning.

Figure 2 shows: Evaluation of strain RCT105 cell fractions containing truncated DBL5 PspA. Proteins from *E. coli* cell fractions were resolved by 10% SDS-PAGE, transferred to NC, and probed with MAb XiR278. Lane 1: molecular weight markers (noted in kDa), lane 2: full-length, native DBL5 PspA, lane 3: uninduced cells, lanes 4-6: induced cells; 1 hr, 2 hr, and 3 hr of IPTG induction respectively, lane 7: periplasmic proteins, lane 8: cytoplasmic proteins, and lane 9: insoluble cell wall/membrane material.

Figure 3 shows: SDS-PAGE of R36A PspA (80 ng) column isolated from CDM-ET and an equal volume of an equivalent WG44.1 prep. Identical gels are shown stained with Bio-Rad silver kit

(A) or immunoblotted with PspA MAb XiR278(B). The PspA isolated from R36A shows the characteristic monomer (84 kDa) and dimer bands.

Figure 4 shows: Cell lysates of pneumococcal isolates MC27 and MC28 were subjected to SDS-PAGE and transferred to nitrocellulose for Western blotting with seven MAb to PspA. 7D2 detected a protein of 82 kDa in each isolate and XiR278 and 2A4 detected a protein of 190 kDa in each isolate. MAb Xi64, Xi126, 1A4 and SR4W4 were not reactive. Strains MC25 and MC26 yielded identical results.

Figure 5 (Figs. 5A and 5B) shows: Southern blot of *Hind* III digest of MC25-MC28 chromosomal DNA developed at a stringency greater than 95 percent. A digest of Rx1 DNA was used as a comparison. The blot was probed with LSM*pspA*13/2, a full length Rx1 probe (Fig. 5) and LSM*pspA*12/6 a 5′ probe of Rx1 *pspA* (Fig. 5). The same concentration of Rx1 DNA was used in both panels, but the concentrations of MC25-MC28 DNA in Fig. 5B were half that used in Fig. 5A to avoid detection of partial digests.

Figure 6 shows: RFLP of amplified *pspA*. *PspA* from MC25 was amplified by PCR using 5′ and 3′ primers for *pspA* (LSM13 and LSM, respectively). The amplified DNA was digested with individual restriction endonucleases prior to electrophoresis and staining with ethidium bromide. Lane 1 *Bcl*I, Lane 2 *BAM*HI, Lane 3 *Bst*NI, Lane 4 *Pst*I, Lane 5 *Sac*I, Lane 6 *Eco*RI, Lane 7 *Sma*I, Lane 8 *Kpn*I.

Figure 7 shows: A depiction of PspA showing the relative location and orientation of the oligonucleotides.

Figure 8 shows: Derivatives of the *S. pneumoniae* D39-Rx1 family.

Figures 9 to 10 show: Electrophoresis of *pspA* or amplified *pspA* product with *Hha*I (Fig. 9), *Sau*3AI (Fig. 10).

Figure 11 shows: RFLP pattern of two isolates from six families.

Figure 12 shows: RFLP pattern of two isolates from six families (using products from amplification with SKH2 and LSM13).

Figure 13 shows: Sequence primarily in the N-terminal half of PspA, Central Region, and Complete Sequence EF5668.

DETAILED DESCRIPTION

Knowledge of and familiarity with the applications incorporated herein by reference is assumed; and, those applications disclose the sequence of *pspA* as well as certain portions thereof, and PspA and compositions containing PspA.

As discussed above and in the following Examples, the invention relates to truncated PspA, e.g., PspA C-terminal to position 192 such as a.a. 192-588 ("BC100") 192-299 and 192-260 of PspA eliciting cross-protection, as well as to DNA encoding such truncated PspA (which amplify the coding for these amino acid regions homologous to most PspAs). The invention further relates to the *pspA*-like gene, or a second *pspA* gene and portions thereof (e.g., probes, primers) which can hybridize thereto

and/or amplify that gene, as well as to DNA molecules which hybridize to *pspA*, so that one can by hybridization assay and/or amplification ascertain the presence of a particular pneumococcal strain; and, the invention provides that a second PspA can be produced by the *pspA*-like sequence (which second PspA can be used like PspA).

Indeed, the invention further relates to oligonucleotide probes and/or primers which react with *pspA* of many, if not all, strains, so as to permit identification, detection or diagnosis of any pneumococcal strain, as well as to expression products of such probes and/or primers, which can provide cross-reactive epitopes.

The repeat region of *pspA* is highly conserved such that the present invention provides oligonucleotide probes or primers to this region reactive with most is not all strains, thereby providing diagnostic assays and a means for identifying epitopes. The invention demonstrates that the *pspA*-like gene is homologous to the *pspA* gene in the leader sequence, first portion of the proline-rich region and in the repeat region; but, these genes differ in the second portion of their proline-rich regions and at the very 3′ end of the gene encoding the 17 amino acid tail of PspA. The product of the *pspA*-like gene is expected to lack a C-terminal tail, suggesting different anchoring than PspA. Drug interference with functions such as surface binding of the coding for repeat regions of *pspA* and the *pspA*-like genes, or with the

repeat regions of the expression products, is therefore a target for intervention of pneumococcal infection. Further still, the invention provides evidence of additional *pspA* homologous sequences, in addition to *pspA* and the *pspA*-like sequence. The invention, as mentioned above, includes oligonucleotide probes or primers which distinguish between *pspA* and the *pspA*-like sequence, e.g., LSM1 and LSM2, useful for diagnostic detecting, or isolating purposes; and LSM1 and LSM10 or LSM1 and LSM7 which amplify a portion of the *pspA*-like gene, particularly the portion of that gene which encodes an antigenic, immunological or protective protein.

The invention further relates to a method for the isolation of native PspA by growth of pneumococcin medium containing high concentrates (about 0.9% to about 1.4%, preferably 1.2%) choline chloride, elation of live pneumococci with a salt solution containing choline chloride, e.g., about 1% about 3%, preferably 2% choline chloride, and growth of pneumococci in medium in which the choline in the medium has been almost or substantially completely replaced with a lower alkanolamine, e.g., C_1-C_6, preferably C_2 alkanolamine, i.e., preferably C_2 alkanolamine, i.e., preferably ethanolamine (e.g., 0.0000005% to 0.0000015%, preferably 0.000001% choline chloride plus 0.02% to 0.04% alkanolamine (ethanolamine), preferably 0.03%). PspA from such pneumococci is then preferably isolated from a choline-sephanrose affinity column, thereby providing

highly purified PspA. Such isolated and/or purified PspA is highly immunogenic and is useful in antigenic, immunological or vaccine composition. Indeed, the growth media of the pneumococci grown in the presence of the alkanolamine (rather than choline) contains PspA and is itself highly immunogenic and therefore useful as an antigenic, immunological or vaccine composition; and, is rather inexpensive to produce. Per microgram of PspA, the PspA in the alkanolamine medium is much more protective than PspA isolated by other means, e.g., from extracts. Perhaps, without wishing to necessarily be bound by any one particular theory, there is a synergistic effect upon PspA by the other components present prior to isolation, or simply PspA is more protective (more antigenic) prior to isolation and/or purification (implying a possibility of some loss of activity from the step of isolation and/or purification).

The invention further relates to the N-terminal 115 amino acids of PspA, which is useful for antigenic, immunological or vaccine compositions, as well as the DNA coding therefor, which is useful in preparing these N-terminal amino acids by recombination, or for use as probes and/or primers for hybridization and/or amplification for identification, detection or diagnosis purposes.

The invention further demonstrates that there is a grouping among the *pspA* RFLP families. This provides a method of identifying families of different PspAs based on RFLP pattern of

pspAs, as well as a means for obtaining diversity of PspAs in an antigenic, immunological or vaccine composition; and, a method of characterizing clonotypes of PspA based on RFLP patterns of PspA. And, the invention thus provides oligonucleotides which permit amplification of most, e.g., a majority, if not all of *S. pneumoriae* and thereby permit RFLP analysis of a majority, if not all, *S. pneumoniae*.

Further, the invention demonstrates that more than one serologically complementary PspA molecule can be in an antigenic, immunological or vaccine composition, so as to elicit better response, e.g., protection, for instance, against a variety of strains of pneumococci; and, the invention provides a system of selecting PspAs for a multivalent composition which includes cross-protection evaluation so as to provide a maximally efficacious composition.

The determination of the amount of antigen, e.g., PspA or truncated portion thereof and optional adjuvant in the inventive compositions and the preparation of those compositions can be in accordance with standard techniques well known to those skilled in the pharmaceutical or veterinary arts. In particular, the amount of antigen and adjuvant in the inventive compositions and the dosages administered are determined by techniques well known to those skilled in the medical or veterinary arts taking into consideration such factors as the particular antigen, the adjuvant (if present), the age, sex, weight, species and

condition of the particular patient, and the route of administration. For instance, dosages of particular PspA antigens for suitable hosts in which an immunological response is desired, can be readily ascertained by those skilled in the art from this disclosure (see, e.g., the Examples), as is the amount of any adjuvant typically administered therewith. Thus, the skilled artisan can readily determine the amount of antigen and optional adjuvant in compositions and to be administered in methods of the invention. Typically, an adjuvant is commonly used as 0.001 to 50 wt% solution in phosphate buffered saline, and the antigen is present on the order of micrograms to milligrams, such as about 0.0001 to about 5 wt%, preferably about 0.0001 to about 1 wt%, most preferably about 0.0001 to about 0.05 wt% (see, e.g., Examples below or in applications cited herein).

Typically, however, the antigen is present in an amount on the order of micrograms to milligrams, or, about 0.001 to about 20 wt%, preferably about 0.01 to about 10 wt%, and most preferably about 0.05 to about 5 wt% (see, e.g., Examples below).

Of course, for any composition to be administered to an animal or human, including the components thereof, and for any particular method of administration, it is preferred to determine therefor: toxicity, such as by determining the lethal dose (LD) and LD_{50} in a suitable animal model e.g., rodent such as mouse; and, the dosage of the composition(s), concentration of components therein and timing of administering the

composition(s), which elicit a suitable immunological response, such as by titrations of sera and analysis thereof for antibodies or antigens, e.g., by ELISA and/or RFFIT analysis. Such determinations do not require undue experimentation from the knowledge of the skilled artisan, this disclosure and the documents cited herein. And, the time for sequential administrations can be ascertained without undue experimentation.

Examples of compositions of the invention include liquid preparations for orifice, e.g., oral, nasal, anal, vaginal, peroral, intragastric, mucosal (e.g., perlingual, alveolar, gingival, olfactory or respiratory mucosa) etc., administration such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration), such as sterile suspensions or emulsions. Such compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON'S PHARMACEUTICAL SCIENCE", 17th edition, 1985,

incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation.

Compositions of the invention, are conveniently provided as liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions or viscous compositions which may be buffered to a selected pH. If digestive tract absorption is preferred, compositions of the invention can be in the "solid" form of pills, tablets, capsules, caplets and the like, including "solid" preparations which are time-released or which have a liquid filling, e.g., gelatin covered liquid, whereby the gelatin is dissolved in the stomach for delivery to the gut. If nasal or respiratory (mucosal) administration is desired, compositions may be in a form and dispensed by a squeeze spray dispenser, pump dispenser or aerosol dispenser. Aerosols are usually under pressure by means of a hydrocarbon. Pump dispensers can preferably dispense a metered dose or, a dose having a particular particle size.

Compositions of the invention can contain pharmaceutically acceptable flavors and/or colors for rendering them more appealing, especially if they are administered orally. The viscous compositions may be in the form of gels, lotions, ointments, creams and the like and will typically contain a sufficient amount of a thickening agent so that the viscosity is from about 2500 to 6500 cps, although more viscous compositions, even up to 10,000 cps may be employed. Viscous compositions have

a viscosity preferably of 2500 to 5000 cps, since above that range they become more difficult to administer. However, above that range, the compositions can approach solid or gelatin forms which are then easily administered as a swallowed pill for oral ingestion.

Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection or orally, to animals, children, particularly small children, and others who may have difficulty swallowing a pill, tablet, capsule or the like, or in multi-dose situations. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with mucosa, such as the lining of the stomach or nasal nucosa.

Obviously, the choice of suitable carriers and other additives will depend on the exact route of administration and the nature of the particular dosage form, e.g., liquid dosage form [e.g., whether the composition is to be formulated into a solution, a suspension, gel or another liquid form], or solid dosage form [e.g., whether the composition is to be formulated into a pill, tablet, capsule, caplet, time release form or liquid-filled form].

Solutions, suspensions and gels, normally contain a major amount of water (preferably purified water) in addition to

the antig n, lipoprotein and optional adjuvant. Minor amounts of other ingredients such as pH adjusters (e.g., a base such as NaOH), emulsifiers or dispersing agents, buffering agents, preservatives, wetting agents, jelling agents, (e.g., methylcellulose), colors and/or flavors may also be present. The compositions can be isotonic, i.e., it can have the same osmotic pressure as blood and lacrimal fluid.

The desired isotonicity of the compositions of this invention may be accomplished using sodium chloride, or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

Viscosity of the compositions may be maintained at the selected level using a pharmaceutically acceptable thickening agent. Methylcellulose is preferred because it is readily and economically available and is easy to work with. Other suitable thickening agents include, for example, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carbomer, and the like. The preferred concentration of the thickener will depend upon the agent selected. The important point is to use an amount which will achieve the selected viscosity. Viscous compositions are normally prepared from solutions by the addition of such thickening agents.

A pharmaceutically acceptable preservative can be employed to increase the shelf-life of the compositions. Benzyl alcohol may be suitable, although a variety of preservatives including, for example, parabens, thimerosal, chlorobutanol, or benzalkonium chloride may also be employed. A suitable concentration of the preservative will be from 0.02% to 2% based on the total weight although there may be appreciable variation depending upon the agent selected.

Those skilled in the art will recognize that the components of the compositions must be selected to be chemically inert with respect to the PspA antigen and optional adjuvant. This will present no problem to those skilled in chemical and pharmaceutical principles, or problems can be readily avoided by reference to standard texts or by simple experiments (not involving undue experimentation), from this disclosure and the documents cited herein.

The immunologically effective compositions of this invention are prepared by mixing the ingredients following generally accepted procedures. For example the selected components may be simply mixed in a blender, or other standard device to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity. Generally the pH may be from about 3 to 7.5. Compositions can be administered in

dosages and by techniques well known to those skilled in the medical and veterinary arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient or animal, and the composition form used for administration (e.g., solid vs. liquid). Dosages for humans or other mammals can be determined without undue experimentation by the skilled artisan, from this disclosure, the documents cited herein, the Examples below (e.g., from the Examples involving mice).

Suitable regimes for initial administration and booster doses or for sequential administrations also are variable, may include an initial administration followed by subsequent administrations; but nonetheless, may be ascertained by the skilled artisan, from this disclosure, the documents cited herein, and the Examples below.

PCR techniques for amplifying sample DNA for diagnostic detection or assay methods are known from the art cited herein and the documents cited herein (see Examples), as are hybridization techniques for such methods. And, without undue experimentation, the skilled artisan can use gene products and antibodies therefrom in diagnostic, detection or assay methods by procedures known in the art.

The following Examples are provided for illustration and are not to be considered a limitation of the invention.

EXAMPLES

EXAMPLE 1 - Truncated Streptococcus pneumoniae PspA Molecules Elicit Cross-Protective Immunity Against Pneumococcal Challenge

Since the isolation of *S. pneumoniae* from human saliva in 1881 and its subsequent connection with lobar pneumonia two years later, human disease resulting from pneumococcal infection has been associated with a significant degree of morbidity and mortality [1]. A recent survey of urgently needed vaccines in the developing and developed world places an improved pneumococcal vaccine among the top three vaccine priorities of industrialized countries [2]. The currently licensed vaccine is a 23-valent composition of pneumococcal capsular polysaccharides that is only about 60% effective in the elderly and due to poor efficacy is not recommended for use in children below two years of age [3]. Furthermore the growing frequency of multi-drug resistant strains of *S. pneumoniae* being isolated accentuates the need for a more effective vaccine to prevent pneumococcal infections.

The immunogenic nature of proteins makes them prime targets for new vaccine strategies. Pneumococcal molecules being investigated as potential protein vaccine candidates include pneumolysis [4], neuraminidase [5], autolysin [5], PspA [6], and PspA [7]. All of these proteins are capable of eliciting immunity in mice resulting in extension of life and protection against death with challenge doses near the LD_{50} [5,8,9]. PspA

is unique among these macromolecules in that is can elicit antibodies in animals that protect against inoculums 100-fold greater than the LD_{50} [10].

PspA is a surface-exposed protein with an apparent molecular weight of 60-99 kDa [11] that is expressed by all clinically relevant *S. pneumoniae* strains examined to date [12]. Though PspAs from different pneumococcal strains are serologically variable, many PspA antibodies exhibit cross-reactivities with PspAs from unrelated strains [12]. Upon active immunization with PspA, mice generate PspA antibodies that protect against subsequent challenge with diverse strains of *S. pneumoniae* [10,7]. The immunogenic and protection-eliciting properties of PspA suggest that it may be a good candidate molecule for a protein-based pneumococcal vaccine.

Four distinct domains of PspA have been identified based on DNA sequence. They include a N-terminal highly charged α-helical region, a proline-rich amino acid stretch, a C-terminal repeat segment comprised of ten 20-amino acid repeat sequences, and a 17-amino acid tail [13]. A panel of MAbs to Rx1 PspA have been produced [14,12,15] and the binding sites of nine of these Mabs were recently localized within the Rx1*pspA* sequence in the α-helical region [15]. Five of the Rx1 Mabs were protective in mice infected with a virulent pneumococcal strain, WU2. Four of these five protective antibodies were mapped to the

distal third (amino acids 192-260) of the α-helical domain of Rx1 PspA [15].

Truncated PspAs containing amino acids 192-588 or 192-299, from pneumococcal strain Rx1 were cloned and the recombinant proteins expressed and evaluated for their ability to elicit protection against subsequent challenge with *S. pneumoniae* WU2 [15]. As with full-length Rx1 PspA [7], both truncated PspAs containing the distal α-helical region protected mice against fatal WU2 pneumococcal infection [15]. However, the recombinant PspA fragment extending from amino acid 192 to 588 was more immunogenic than the smaller fragment, probably due to its larger size. In addition, the protection elicited by the amino acid fragment 192-588 of Rx1 was comparable to that elicited by full-length Rx1 PspA [15]. Therefore, cross-protective epitopes of other PspAs were also sought in the C-terminal two-thirds of the molecule. As discussed below, PspAs homologous to amino acids 192-588 of strain Rx1 were amplified by PCR, cloned, and expressed in *E. coli*. Then three recombinant PspAs, from capsule type 4 and 5 strains, were evaluated for their ability to confer cross-protection against challenge strains of variant capsular types. The data demonstrate that the truncated PspAs from capsular type 4 and 5 strains collectively protect against or early death caused by challenge with capsular type 4 and 5 parental strains as well as type 3, 6A, and 6B *S. pneumoniae*.

Bacterial strains and culture conditions. All pneumococci were from the culture collection of this laboratory and have been previously described [16,17,18,19] with the exception of clinical isolates TJ0893, 0922134, and BG8740. Pneumococcal strains TJ0893 and 0922134 were recovered from the blood of a 43-year old male and an elderly female, respectively. *S. pneumoniae* BG8743 is a blood isolate from an 8-month old infant. Strains employed in this study included capsular type 3 (A66.3, EF10197, WU2), type 4 (BG9739, EF3296, EF5668, L81905), type 5 (DBL5), type 6A (DBL6A, EF6796), type 6B (BG7322, BG9163, DBL1), type 14 (TJ0893), type 19 (BG8090), and type 23 (0922134, BG8743). In addition strain WG44.1 [10], which expresses no detectable PspA, was employed in PspA-specific antibody analysis. All chemicals were purchased from Fisher Scientific, Fair Lawn, New Jersey unless indicate otherwise.

S. pneumoniae were grown in Todd Hewitt broth (Difco, Detroit, Michigan) supplemented with 5% yeast extract (Difco). Mid-exponential phase cultures were used for seeding inocula in Lactated Ringer's (Abbott laboratories, North Chicago, Illinois) for challenge studies. The LD_{50s} were previously determined [16,20]. For pneumococcal strains used in challenge studies, inocula ranged from 2.8 to 3.8 $\log_{10}$ CFU (verified by dilution plating on blood agar). Plates were incubated overnight in a candle jar at 37°C.

E. coli DH1 and BL21(DE3) were cultured in LB medium (1% Bacto-tryptone (Difco), 0.5% Bacto Yeast (Difco), 0.5% NaCl, 0.1% dextrose). For the preparation of cell lysates, recombinant *E. coil* were grown in minimal E medium [21] supplemented with 0.05 M thiamine, 0.2% glucose, 0.1% casamino acids (Difco), and 50 mg/ml kanamycin. Permanent bacterial stocks were stored at -80°C in growth medium containing 10% glycerol.

Construction of plasmid-based strains. pET-9a (Novagen, Madison, Wisconsin) was used for cloning truncated *pspA* genes from fourteen *S. pneumoniae* strains: DBL5, DBL6A, WU2, BG9739, EF5668, L81905, 0922134, BG8090, BG8743, BG9163, DBL1, EF3296, EF6796, and EF10197 (Table 1). *pspA* gene fragments, from fifteen strains, were amplified by PCR using two primers provided by Connaught Laboratories, Swiftwater, Pennsylvania Primer N192-5'GGAAGGCCATATGCTCAAAGAGATTGATGAGTCT3' and primer C588 - 5'CCAAGGATCCTTAAACCCATTCACCATTGGC3' were engineered with *Nde*I and *Bam*HI restriction endonuclease sites, respectively. PCR-amplified gene products were digested with *Bam*HI and *Nde*I, and ligated to linearized pET-9a digested likewise and further treated with bacterial alkaline phosphatase United States Biochemical Corporation, Cleveland, Ohio) to prevent recircularization of the cut plasmid. Clones were first established in *E. coli* BL21(DE3) which contains a chromosomal copy of the T7 RNA polymerase gene under the control of an inducible *lacUV5* promoter [22].

E. coli DH1 cells were transformed by the method of Hanahan [23]. Stable transformants were identified by screening on LB-kanamycin plates. Plasmid constructs, isolated from each of these strains, were electroporated (Electro Cell Manipulator 600, BTX Electroporation System, San Diego, California) into *E. coli* BL21(DE3) and their respective strain designations are listed in Table 1. The pET-9a vector alone was introduced into *E. coli* BL21(DES) by electroporation to yield strain RCT125 (Table 2). All plasmid constructs and PCR-amplified *pspA* gene fragments were evaluated by agarose gel electrophoresis (with 1 kb DNA ladder, Gibco BRL, Gaithersburg, Maryland). Next, Southern analysis was performed using LM*pspA*1, a previously described full-length *pspA* probe [18] random primed labeled with digoxigenin-11-dUTP (Genius System, Boehringer Mannheim, Indianapolis, Indiana). Hybridization was detected with chemiluminescent sheets as per manufacturer's instructions (Schleicher & Schuell, Keene, New Hampshire).

Cell fractionation of recombinant *E. coli* strains. Multiple cell fractions from transformed *E. coli* were evaluated for the expression of truncated PspA molecules. Single colonies were inoculated into 3 ml LB cultures containing kanamycin and grown overnight at 37°C. Next, an 80 ml LB culture, inoculated with 1:100 dilution of the overnight culture, was grown at 37°C to mid-exponential phase (A_{600} of ca. 0.5) and a 1 ml sample was harvested and resuspended (uninduced cells) prior to induction

with isopropylthiogalactoside (IPTG, 0.3 mM final concentration). Following 1, 2, and 3 hr of induction, 0.5 ml of cells were centrifuges, resuspended, and labeled induced cells. The remaining culture was divided into two aliquots, centrifuged (4000 x g, 10 min, DuPont Sorvall RC 5B Plus), and the supernatant discarded. One pellet was resuspended in 5 ml of 20 mM Tris-HC1 ph 7.4 200 Mm NaC1, 1 Mm (ethylenedinitrilo)-tetraacetic acid disodium salt (EDTA) and frozen at -20°C overnight. Cells were thawed at 65°C for 30 min, placed on ice, and sonicated for vive 10-sec pulses (0.4 relative output, Fisher Sonic Dismembrator, Dynatech Laboratories, Inc. Chantilly, Virginia). Next, the material was centrifuged (9000 x g, 20 min) and the supernatant was designed the crude extract-cytoplasmic fraction. The pellet was resuspended in Tris-NaC1-EDTA buffer and labeled the insoluble cell well and membrane fraction. The other pellet, from the divided induced culture, was resuspended in 10 ml of 30 mM Tris-HC1 pH 8.0 containing 20% sucrose and 1 mM EDTA and incubated at room temperature for 10 min with agitation. Cells were then centrifuged, the supernatant removed, and the pellet resuspended in 5 mM $MgSO_4$ (10 ml, 10 min, shaking 4°C bath). This material was centrifuged and the supernatant was designated osmotic shock-periplasmic fraction. Cell fractions were evaluated by SDS-PAGE and immunoblot analysis.

MAbs to PspA. PspA-specific monoclonal antibodies (MAbs) XiR278 and 1A4 were previously described [12]. MAb P50-

92D9 was produced by immunization with DBL5 PspA (kindly provided by Dr. Robert Becker, Connaught Laboratories, Swiftwater, Pennsylvania). The PspA-specificity of MAb P50-92D9 was confirmed by Western Analysis by its reactivity with native PspAs from *S. pneumoniae* DBL5, BG9739, EF5668, and L81095 and its failure to recognize the PspA-control strain WG44.1.

SDS-PAGE and immunoblot analysis. *E. coli* cell fractions containing recombinant PspA proteins and biotinylated molecular weight markers (low range, Bio-Rad, Richmond, California) were separated by sodium dodecyl sulfate-polyacrylamide (10%; Bethesda Research Laboratories, Gaithersburg, Maryland) gel electrophoresis (SDS-PAGE) by the method of Laemmli [24]. Samples were first boiled for 5 min in sample buffer containing 60 mM Tris pH 6.8, 1% 2-B-mercaptoethanol (Sigma, St. Louis, Missouri), 1% SDS, 10% glycerol, and 0.01% bromophenol blue. Gels were subsequently transferred (1 hr, 100 volts) to nitrocellulose (0.45 mM pores, Millipore, Bedford, Massachusetts) as per the method of Towbin et al. [25]. Blots were blocked with 3% casein, 0.05% Tween 20 in 10 mM Tris, 0.1 M NaCl, pH 7.4 for 30 min prior to incubating with PspA-specific monoclonal antibodies diluted in PBST for 1 hr at 25°C. Next, the blot was washed 3 times with PBST before incubating with alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Inc., Birmingham, Alabama) for 1 hr at 25°C. Washes were performed as

before and blots was developed with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.01% nitro blue tetrazolium (Sigma) first dissolved in 150 μl of dimethyl sulfoxide and then diluted in 1.5 M Tris-HCl pH 8.8. Dot blots were analyzed similarly: Lysate samples (2 μl) were spotted on nitrocellulose filters (Millipore), allowed to dry, blocked, and detected as just described.

Preparation of cell lysates containing recombinant PspA proteins. Transformed *E. coli* strains RCT105, RCT113, RCT117, and RCT125 (Table 2) were grown in mid-exponential phase in minimal E medium before IPTG induction (2 mM final concentration, 2 hours, 37°C). Cultures were harvested by centrifugation (10 min at 9000 x g), resuspended in Tris-acetate pH 6.9, and frozen at -80°C overnight. Samples were thawed at 65°C for 30 min, cooled on ice, sonicated as previously described. Next the samples were treated with 0.2 mM AEBSF (Calbiochem, La Jolla, California) at 37°C for 30 min and finally centrifuged to remove cell wall and membrane components. Dot blot analysis was performed using PspA-specific MAbs to validate the presence of recombinant, truncated PspA molecules in the lysates prior to their use as immunogens in mice. Unused lysate material was stored at -20°C until subsequent immunizations were performed.

Mouse immunization and challenge. CBA/CAHN-XID/J mice (Jackson Laboratories, Bar Harbor, Maine), 6-12 weeks old, were employed for protection studies. These mice carry a X-linked

immunodeficiency that prevents them from generating antibody to polysaccharide components, thus making them extremely susceptible to pneumococcal infection [26,27]. Animals were immunized subcutaneously with cell lysates from *E coli* recombinant strains RCT105, RCT113, RCT117, and RCT125 (Table 2) in complete Freund's adjuvant for primary immunizations. Secondary injections were administered in incomplete adjuvant and subsequent boosts in dH_2O. Immunized and nonimmunized mice (groups of 2 to 5 animals) were challenged with *S. pneumoniae* strains A66.3, BG7322, DBL6A, WU2, DBL5, BG9739, and L81905 intravenously (tail vein) to induce pneumococcal sepsis. Infected animals were monitored for 21 days and mice that survived the 3-week evaluation period were designated protected against death and scored as surviving 22 days for statistical analysis. Protection that resulted in extension of life was calculated as a comparison between mean number of days to death for immunized versus pooled control mice (nonimmunized and RCT125 sham-immunized; total of 6-7 animals).

Determination of PspA serum levels. Mice were bled retro-orbitally following the secondary boost and again prior to challenge. Representative mouse titers were evaluated by enzyme-linked immunorsorbent assay (ELISA) using native, parental PspAs isolated from pneumococcal strains DBL5, BG9739, and L81905. PspAs were immobilized on microtiter plates by incubating in 0.5 $NaHCO_3$, 0.5 M Na_2CO_3pH9.5 at 4°C overnight. Alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Southern Biotechnology

Associates, Inc.) was used to detect mouse serum antibodies. Color development was with p-nitrophenyl phosphate (Sigma, 1 mg/ml) in 0.5 m $MgCL_2$ pH 9.8 with 10% diethanolamine and absorbance was read at 405 nm after a 30 min incubation. Reciprocal titers were calculated as the last dilution of antibody that registered an optical density value of 0.1. Sera from individual mice within a particular immunogen group were evaluated separately and then the respective titers from four mice per group were combined to obtain titer range (Table 3).

Statistics. The one-tailed Fisher exact and two sample rank tests [28] were used to evaluate protection against death and extension of life in the mouse model.

Cloning of truncated *pspA* genes. Using primers N192 and C588, truncated *pspA* genes from fifteen diverse pneumococcal strains representing eight different capsular types (Table 1) were amplified by PCR. Even though variability exists in *pspA* genes from different strains [18], this result demonstrates that sufficient conversation exists between variant *pspA* genes to allow sequence amplification in all strains examined to date. Successful *pspA* PCR-amplification extended to all capsule types evaluated.

Fourteen of the amplified *pspA* genes were cloned and three clones containing truncated PspA molecules from pneumococcal strains DBL5, BG9739, and L81905 were further studies (Table 2). To verify the constructions, plasmids from

recombinant *E. coli* strains (RCT105, RCT113, RCT117, and RCT125 (Table 2) were isolated, digested with *Nde*I and *BAM*HI restriction endonucleases, and electrophoresed in 1% agarose side-by-side with the PCR products used in their respective constructions (Figure 1A). The digestion reaction was complete for pRCT105, while pRCT113 and pRCT117 digestions were incomplete (lanes 5 and 7, respectively). This gel was denatured and DNA transferred to nylon for Southern analysis. Figure 1B depicts the corresponding Southern blot probed with full-length Rx1*pspA* DNA. Lane 1 contains pRCT125, digested vector alone, which does not react with the pneumococcal DNA-specific probe, as expected. the *pspA*-specific probe hybridized with the PCT products and the digested plasmid inserts (see arrow, Figure 1B) as well as the partially undigested pRCT113 and pRCT117 (lane 5 and 7), confirming successful cloning of DBL5, BG9739, and L81905 *pspA* DNA. Constructions were similarly confirmed with the eleven additional recombinant strains containing truncated *pspA* genes from *S. pneumoniae* strains of different capsular and PspA types.

Expression of recombinant PspA in *E. coli* Bl21(De3).

Transformed *E. coli* strains RCT105, RCT113, RCT117, and RCT125 were cultured to mid-exponential phase prior to the addition of IPTG to induce expression of the cloned, truncated *pspA* gene in each strain. A cell fractionation experiment was performed to identify the location of recombinant PspA proteins in transformed *E. coli* strains. Samples representing uninduced cells, included

cells (1 hr, 2 hr, and 3 hr time intervals), the periplasmic fraction, the cytoplasmic fraction, and insoluble cell wall/membrane material were resolved by SDS-PAGE. Proteins were then transformed to introcellulose and Western analysis was performed using monoclonal antibodies specific for PspA epitopes.

Figure 2 reveals that both the cytoplasmic (lane 8) and the insoluble matter fractions (lane 9), from recombinant strain RCT 105, contain a protein of approximately 53.7 kDa that is recognized by MAb XiR278 that is not seen in the uninduced cell sample (lane 3). This protein increases in quantity in direct correlation with the length of IPTG induction (lanes 4-6; 1 hr, 2 hr, and 3 hr respectively). No truncated RCT105 PspA was found in the periplasmics fraction (lane 7), which was expected since the pET-9a vector lacks a signal sequence that would be necessary for directing proteins to the periplasm. The observed molecular weight (ca. 53.5 kDa) is larger than the predicted molecular weight for the 1.2 kb DBL5*pspA* gene product (43.6 kDa; Figure 1A, lane 4). Like full-length Rx1 PspA (Yother, 1992), the observed and predicted molecular weights for truncated PspAs do not agree precisely. In addition, immunoblot analysis was performed for recombinant *E. coli* strains RCT113, and RCT117 (using MAbs 1A4 and P50-92D, respectively) and similar results were obtained, while no cell fractions from control strain RCT125 were recognized by MAb XiR278.

Evaluating the protective capacity of recombinant, truncated PspAs. The truncated PspA proteins from strains RCT113, RCT117, and RCT105 were expressed and analyzed for their ability to generate cross-protection against a battery of seven *S. pneumoniae* strains. Control mice (non-immunized and RCT125 sham-immunized) and recombinant PspA-immunized mice were challenged with mouse-virulent strains A66.3, BG7322, DBL6A, WU2, DBL5, BG9739, and L81905. Table 3 presents the day of death for each infected mouse.

Immunization with truncated PcpA from RCT113, RCT117, and RCT105 conferred protection against death for all mice challenged with capsular type 3 strains (A66.3 and WU2 (Table 3). The three truncated PSpAs also provided significant protection against death with DBL6A, and BG7322 pneumococci (capsular types 6A and 6B, respectively). In addition, immunization with recombinant RCT113 PspA extended days to death in mice challenged with strains DBL5, BG9739, and L81905, while RCT117 PspA prolonged the lives of mice inoculated with BG9739 pneumococci (Table 3). Truncated BG9739 PspA elicited protection against all challenge strains (100%) evaluated in this study, while recombinant L81905 and DBL5 truncated PspAs conferred protection against death with 71% and 57% of *S. pneumoniae* challenge strains, respectively.

Anti-PspA antibody titers elicited by the three immunogens vary over approximately a 10-fold range (Table 3).

The lowest antibody levels were elicited by RCT105 and this truncated PspA also elicited protection against the fewest number of challenge strains. RCT113 and RCT117 elicited three and nine time as much anti-PspA antibody, respectively. As expected, no antibody to PspA was detected in nonimmunized mice nor was specific-PspA antibody measured in mice immunized with the vector-only control strain (RCT125).

In summary, immunization with RCT113 and RCT117 PspAs protected mice against fatal challenge with capsular type 3 and 6A strains and extended life for mice inoculated with type 4, 5, and 6B pneumococci. RCT105 PspA immunization protected against fatal infection with capsular type 3 and 6B strains and prolonged time to death for type 6A *S. pneumoniae* but offered not protection against type 4 and 5 strains. These data demonstrate that truncated PspAs from capsular type 4 and 5 pneumococci collectively protect mice and ergo other hosts, such as humans, against or delay death caused by each of the seven challenge strains. In general, however, more complete protection was observed against strains of capsular type 3, 6A, and 6B than against type 4 and 5 *S. pneumoniae*.

PspA has been shown to be a protection-eliciting molecule of *S. pneumoniae* [10,7,29]. Immunization with PspA has also been shown to be cross-protective, although eliciting more complete protection against certain strains than others [7]. Thus, it is possible that a broadly protective PspA vaccine might

need to contain PspAs of more than one pneumococcal strain. The distal third of the α-helical region of PspA has been identified as a major protective region of PspA [15]. Moreover, this region is presented in a very antigenic form when expressed with the intact C-terminal half of the molecule [15]. In this Example, the ability to use truncated PspA proteins homologous to the region of Rx1 PspA extending from amino acid residue 192 to the C-terminus at residue 588 is demonstrated.

The C-terminal two-thirds of PspA was cloned from fourteen strains by PCR amplification of a gene fragment of the appropriate size (1.2 kb) which hybridized with full-length Rx1 *pspA*. Successful PCR amplification extended to all capsule types analyzed. Thus, the C-terminal two-third of PspA may be amplified from many, if not all, pneumococcal capsule types with Rx1 *pspA*-specific primers. This technique is thus applicable to the development of antigenic immunological or vaccine compositions containing multiple PspA or fragments thereof.

Of these clones, three truncated PspA proteins were expressed and evaluated in mouse immunization studies to determine their ability to cross-protect against challenge with a variety of pneumococcal capsular types. All three recombinant PspAs elicited antibody reactive with their respective donor PspA and all three elicited protection against pneumococcal infection. Of the two truncated PspA proteins that elicited the highest antibody responses, 100% and 71% of the challenge strains were

protected. RCT105 PspA, which elicited the lowest titers of PspA-specific antibody, yielded protection against 57% of *S. pneumoniae* strains evaluated. With all truncated PspAs, significant levels of protection were observed in four of the seven challenge strains. In fact, in all instances except for on (RCT105-immunized mice challenged with strain BG9739) the trend was for truncated PspA-immunization to elicit protection against pneumococcal challenge. These results demonstrate that truncated Rx1 PspA (amino acids 192-588) cross-protects mice against fatal *S. pneumoniae* WU2 challenge. More importantly, these data show that the homologous regions of diverse PspAs demonstrate comparable cross-protective abilities.

Strains of capsular type 4 and 5 were more difficult to protect against than were type 3, 6A and 6B pneumococcal strains. Serological differences in PspAs might affect cross-protection in some cases. Yet the difficulty in protecting against the type 4 and 5 strains used herein could not be explained on this basis, since the truncated PspA immunogens were cloned from the same three type 4 and 5 strains used for challenge. Both PspAs from the type 4 strains delayed death caused by one or both type 4 challenge strains but neither could prevent death caused by either type 4 pneumococcal strain. Moreover, the truncated PspA from the type 5 strain DBL5 elicited protection against death or delayed death with strains of capsular types 3, 6A and 6B but

failed to protect against infection with its donor strain or either type 4 challenge strain.

There may be several reasons why the truncated PspAs from capsular type 4 and type 5 strains failed to protect against death even with their homologous donor *S. pneumoniae* strains. One possibility is that the type 4 and 5 strains chosen for study are especially virulent in the XID mouse model. XID mice fail to make antibodies to polysaccharides [26,27] and are therefore extremely susceptible to pneumococcal infection with less than 100 CFU of most strains, including those of capsular type 3, 4, 5, 6A, and 6B. The increased mouse virulence of types 4 and 5 is apparent from the fact that in immunologically normal mice these strains have lower LD_{50}s and/or are more consistently fatal than strains of capsular types 3, 6A, or 6B [17].

Another possibility is that epitopes critical to protection-eliciting capacity with capsular type 4 and 5 strains are not present in the C-terminal two-thirds of PspA (amino acids 192-588), the truncated fragments used for immunization. The critical epitopes for these strains may be located in the N-terminal two thirds of the α-helical region of their PspA molecules. Finally, it is also possible that PspA may be less exposed on some *S. pneumoniae* strains than others. Strain Rx1 PspA amino acid sequence does not contain the cell wall attachment motif LPXTGX described by Schneewind et al [30] found in many gram-positive bacteria. Rather, PspA has a novel

anchoring mechanism that is mediated by choline interactions between pneumococcal membrane-associated lipoteichoic acid and the repeat region in the C-terminus of the molecule [31]. Electron micrographic examination has confirmed the localization of PspA on the pneumococcal surface [32] and PspA-specific MAb data supports the accessibility of surface-exposed PspA. However, it is not known whether *S. pneumoniae* strains differ substantially in the degree to which different PspA regions are exposed to the surrounding environment. Nor is it known if the quantity of PspA expressed on the bacterial cell surface differs widely between strains.

Table 1. pspA recombinant strains categorized by pneumococcal capsular type.

Capsular Type	Parent Strains	Respective Recombinant Strains
3	WU2, EF10197	RCT111, RCT137
4	BG9739, EF5668	RCT113, RCT115
	L81905, EF3296	RCT117, RCT133
5	DBL5	RCT105
6A	DBL6A, EF6796	RCT109, RCT135
6B	BG9163, DBL1	RCT129, RCT131
14	TJO893	none*
19	BG8090	RCT121
23	0922134, BG8743	RCT119, RCT123

* Truncated pspA amplified recently, not yet cloned

Table 2. Description of recombinant strains used in evaluating the protection-eliciting capacity of truncated PspAs in mice.

Recombinant Strain	Description	Capsule Type of Parent PspA
RCT 105	BL21(DE3) E. coli with pET-9a:DBL5	5
RCT 113	BL21(DE3) E. coli with pET-9a:BG9739	4
RCT 117	BL21(DE3) E. coli with pET-9a:L81905	4
RCT 125	BL21(DE3) E. coli with pET-9a (vector only)	

Table 3. Evaluation of the protection elicited by truncated S. pneumoniae PspA molecules in mice by days to death post-challenge*.

		Challenge Strain [capsular type] (log_{10} dose in CFU)						
Immunizing recombinant PspA/ PspA donor strain	Reciprocal anti-PspA titer†	A66.3 [type 3] (2.78)	WU2 [type 3] (3.57)	DBL6A [type 6A] (3.24)	BG7322 [type 6B] (3.11)	DBL5 [type 5] (3.81)	BG9739 [type 4] (3.56)	L81905 [type 4] (3.62)
RCT113/BG9739	5590 - 50,300	4x >21 ‡	4x >21 §	15, 3x >21 ‡	12,13,16,>21 ‡	3, 3, 4, 5 §	5, 5, 5, 7 §	5, 6, 8, 8 ‡
RCT117/L81905	5590 - 150,900	4x >21 ‡	4x >21 §	7, 16, 2x >21 ‡	10,12,13,>21 §	3, 3, 4, 4 ¶	4, 5 ,13, >21§	3, 4, 6, 8
RCT105/DBL5	1860 - 16,770	4x >21 ‡	4x >21 §	8, 10, 13, 21 ‡	4x >21 ‡	2, 2, 2 ,>21	2, 2, 2, 4	4, 5, 5, 5
RCT125/vector only	20 - 620	3, 6, 6, >21	2, 3, 3, >21	3, 6, 6, 6	7, 8, 8, 14	2, 2, 2, 2	2, 2, 3, 4, 5	2, 3, 5, 5
none	0	2, 2, 2	2, 3	3, 3, 4	6, 7, 9	2, 5	3, 5	2, 5

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* Animals surviving the 3-week evaluation period were sacrificed and days to death recorded as >21 days. For statistical analysis, P values were calculated at 22 days for these fully protected mice.

† Range of four sera per group of mice; titers measured against native donor PspAs

‡ $P \leq 0.012$

§ $P \leq 0.035$

¶ $P \leq 0.057$

Note: One-tailed Fisher exact and two sample rank tests were used for statistical analysis.

Table 3. Evaluation of the protection elicited by truncated S. pneumoniae PspA molecules in mice by days to death post-challenge*.

		Challenge Strain [capsular type] (log_{10} dose in CFU)						
Immunizing recombinant PspA/ PspA donor strain	Reciprocal anti-PspA titer†	A66.3 [type 3] (2.78)	WU2 [type 3] (3.57)	DBL6A [type 6A] (3.24)	BG7322 [type 6B] (3.11)	DBL5 [type 5] (3.81)	BG9739 [type 4] (3.56)	L81905 [type 4] (3.62)
RCT113/BG9739	5590 - 50,300	4x >21 ‡	4x >21 §	15, 3x >21 ‡	12,13,16,>21 ‡	3, 3, 4, 5 §	5, 5, 5, 7 §	5, 6, 8, 8 ‡
RCT117/L81905	5590 - 150,900	4x >21 ‡	4x >21 §	7, 16, 2x >21 ‡	10,12,13,>21 §	3, 3, 4, 4 ¶	4, 5 ,13, >21§	3, 4, 6, 8
RCT105/DBL5	1860 - 16,770	4x >21 ‡	4x >21 §	8, 10, 13, 21 ‡	4x >21 ‡	2, 2, 2 ,>21	2, 2, 2, 4	4, 5, 5, 5
RCT125/vector only	20 - 620	3, 6, 6, >21	.2, 3, 3, >21	3, 6, 6, 6	7, 8, 8, 14	2, 2, 2, 2	2, 2, 3, 4, 5	2, 3, 5, 5
none	0	2, 2, 2	2, 3	3, 3, 4	6, 7, 9	2, 5	3, 5	2, 5

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* Animals surviving the 3-week evaluation period were sacrificed and days to death recorded as >21 days. For statistical analysis, P values were calculated at 22 days for these fully protected mice.

† Range of four sera per group of mice; titers measured against native donor PspAs

‡ $P \leq 0.012$

§ $P \leq 0.035$

¶ $P \leq 0.057$

Note: One-tailed Fisher exact and two sample rank tests were used for statistical analysis.

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EXAMPLE 2 - Localization of prot ction-eliciting pitopes and PspA of *S. pneumoniae*

This Example, the ability of PspA epitopes on two PspA fragments (amino acids 192-588 and 192-299) to elicit cross-protection against a panel of diverse pneumococci is demonstrated. Also, this Example identifies regions homologous to amino acids 192-299 of Rx1 in 15 other diverse pneumococcal strains. The DNA encoding these regions was then amplified and cloned. The recombinant PspA fragments expressed were evaluated for their ability to elicit cross-protection against a panel of virulent pneumococci.

Bacterial strains and media conditions. *S. pneumoniae* strains were grown in Todd Hewitt broth with 0.5% yeast extract (THY) (both from Difco Laboratories, Detroit, Michigan) at 37°C or on blood agar plates containing 3% sheep blood at 37°C under reduced oxygen tension. *E. coli* strains were grown in Luria-Bertani medium or minimal E medium [Davis, 1980 #143]. Bacteria were stored at -80°C in growth medium supplemented with 10% glycerol. *E. coli* were transformed by the methods of Hanahan [Hanahan, 1983]. Ampicillin (Ap) was used at a concentration of 100 μg/ml for *E. Coli*.

Construction of pIN-III-ompA3 and pMAL-based *E. Coli* strains. Recombinant plasmids pBC100 and pBAR416 that express and secrete *pspA* fragments from *E. Coli* were constructed with pIN-III-ompA3 as previously described [McDaniel, 1994].

The pMAL-p2 vector (New England Biolabs, Protein Fusion & Purification System, catalog #800) was used for cloning *pspA* gene fragments to amino acids 192-299 from strain Rx1 and from 7 other *S. pneumoniae* strains: R36A, D39, A66, BF9739, DBL5, DBL6A, and LM100. Amplification of the *pspA* gene fragments was done by the polymerase chain reaction (PCR) as described previously [McDaniel, 1994] using primers 5'CCGGATCCGCTCAAAGAGATTGATGAGTCTG3' [LSM4] and 5'CTGAGTCGACTGAGTTTCTGGAGCTGGAGC3' [LMS6] made with *Bam*HI and *Sal*I restriction endonuclease sites, respectively. Primers were based on the sequence of Rx1 PspA [Yother, 1992]. PCR products and the pMAL vector were digested with *BAM*HI and *Sal*I, and ligated together. Clones were transformed into *E. Coli* DH5α by the methods of Hanahan [Hanahan, 1983]. Stable transformants were selected on LB plates containing 100μg/ml Ap. These clones were screened on LB plates containing 0.1 mM IPTG, 80 μg/ml X-gal and 100 μg/ml Ap and replica LB plates with 100 μg/ml Ap as per manufacturer's instructions. The strain designations for these constructs are listed in Table 6. Positive clones were evaluated for the correct *pspA* gene fragment by agarose gel electrophoresis following plasmid isolation by the methods of Birnboim and Doly [Birnboim, 1979]. Southern analysis was done as previously described [McDaniel, 1994] using a full-length *pspA* probe randomly primed labeled with digoxigenin-11-dUTP (Genius System,

Boehdinger Mannheim, Indianapolis, Indiana) and detected by chemiluminescence.

Expression of recombinant PspA protein fragments. For induction of expression of strains BC100 and BAR416, bacteria were grown to an optical density of approximately 0.6 at 660 nm at 37°C in minimal media, and IPTG was added to a final concentration of 2 mM. The cells were incubated for an additional 2 hours at 37°C, harvested, and the periplasmic contents released by osmotic shock [Osborn, 1974 #100]. For strains BAR36A, BAR39, BAR66, BAR5668, BAR9739, BARL5, BAR6A and BAR100, bacteria were grown and induced as above except LB media + 10 mM. glucose was the culture medium. Proteins from these strains were purified over an amylose resin column as per manufacturer's instructions (New England Biolabs, Protein Fusion & Purification System, Catalog #800). Briefly, amylose resin was poured into a 10 mL column and washed with column buffer. The diluted osmotic shock extract was loaded at a flow rate of approximately 1 mL/minute. The column was then washed again with column buffer and the fusion protein eluted off the column with column buffer + 10 mM maltose. Lysates were stored at -20°C until further use.

Characterization of truncated PspA proteins used for immunization. Immunoblot analysis was carried out as described previously (McDaniel, 1991 #14). Briefly, the truncated PspA mol cules, controls and molecular weight markers (Bio-Rad,

Richmond, CA) were electrophoresed in a 10% sodium dodecyl (SDS) - polyacrylamide gel and electroblotted onto nitrocellulose. Rabbit polyclonal anti-PspA serum and rabbit antimaltose binding protein were used as the primary antibodies to probe the blots.

A direct binding ELISA procedure was used to quantitatively confirm reactivities observed by immunoblotting. For all protein extracts, osmotic shock preparations were diluted to a concentration of 3 μg/ml in phosphate buffered saline (PBS), and 100 μl was added to the wells of Immulon 4 microtitration plates (Dynatech Laboratories, Inc., Chantilly, VA). After blocking with 1.5% bovine serum albumin in PBS, unfactionated tissue culture supernates of individual MAbs were titered in duplicated by three-fold serial dilution through seven wells and developed using an alkaline phosphatase-labeled goat anti-mouse immunoglobulin secondary antibody (Southern Biotech Associates, Birmingham, AL) and alkalinephosphatase substrate (Sigma, St. Louis, MO) as previously described (McDaniel, 1994). The plates were read at 405 nm in a Dynatech plate reader after 25 minutes, and the 30% end point was calculated for each antibody with each preparation.

Immunization and Protection Assays. Six to nine week old CBA/CAHN-XID/J (CBA/N) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. CBA/N mice carry an X-linked immunodeficiency trait, which renders them relatively unable to respond to prolysaccharide antigens, but they do respond with

normal levels of antibodies against protein antigens (Amsbaugh, 1972; Wicker, 1986). Because of the absence of antibodies reactive with the phosphocholine determinant of C-polysaccharide in their serum, the mice are highly susceptible to pneumococcal infection (Briles, 1981). Mice immunized with the BC100 fragment were injected inguinally with antigen emulsified in CFA, giving an approximate dose of 3 μg of protein per mouse. Fourteen days later the mice were boosted intraperitoneally with 3 μg of antigen diluted in Ringer's lactate without adjuvant. Control mice were immunized following the same protocol with diluent and adjuvant, but no antigen. Mice immunized with the BAR416 fragment were injected with 0.2 ml at two sites in the subinguinal area with antigen emulsified in CFA. The mice were boosted inguinally fourteen days later with antigen emulsified in IFA and were boosted a second time fourteen days later intraperioneally with 0.2 ml of antigen diluted in Ringer's lactate without adjuvant.

Mice that were immunized with the homologues of Rx1 BAR416 were immunized as described above. The control animals followed the same immunization protocol but received maltose binding protein (MBP) diluted 1:1 in CFA for their immunization and were also boosted with MBP.

Serum analysis. Mice were retro-orbitally bled with a 75 μl heparinized microhematocrit capillary tube (Fisher Scientific) befor the first immunization and then once

approximat ly 2 hours before challenge with virulent pneumococci. The serum was analyzed for the presence of antibodies to PspA by an enzyme-linked immunosorbent assay (ELISA) using native full-length R36A PspA as coating antigen as previously described (McDaniel, 1994).

Intravenous infection of mice. Pneumococcal cultures were grown to late log phase in THY. Pneumococci were diluted to 10^4 CFU based on the optical density at 420 nm into lactated Ringer's solution. Seven days following the last boost injection for each group, diluted pneumococci were injected intravenously (tail vein) in a volume of 0.2 ml and plated on blood agar plates to confirm the numbers of CFU per milliliter. The final challenge dose was approximately 50-100 times the LD_{50} of each pneumococcal strain listed in Tables 4-6. The survival of the mice was followed for 21 days. Animals remaining alive after 21 days were considered to have survived the challenge.

Statistical analysis. Statistical significance of differences in days to death was calculated with the Wilcoxon two-sample rank test. Statistical significance of survival versus death was made using the Fisher exact test. In each case, groups of mice immunized with PspA containing preparations were compared to unimmunized controls, or controls immunized with preparations lacking PspA. One-tailed, rather than two-tailed, calculations were used since immunization with PspA or fragments

of PspA has never been observed to cause a statistically significant decrease in resistance to infection.

Cloning into pMAL vector. Using primers based on the sequence of Rx1 PspA, LSM4 and LSM6, *pspA* gene fragments were amplified by PCR from fifteen out of fifteen pneumococcal strains examined. Seven of the eleven gene fragments were cloned into pMAL-p2 and transformed into *E. coli* (Table 6). The correct insert for each new clone was verified by agarose gel electrophoresis and Southern hybridization analysis. Plasmids from recombinant *E. coli* strains BAR36A, BAR39, BAR66, BAR9739, BARL5, BAR6A and BAR100 were isolated, digested with *Bam*HI and *Sal*I restriction endonucleases and electrophoresed on a 0.7% TBE agarose gel. The gel was then denatured and the DNA transferred to a nylon membrane for southern hybridization. The blot was probed with full-length Rx1 *pspA* DNA at high stringency conditions. The cloning of R36A, D39, A66, BG9739, DBL5, DBL6A and LM100 *pspA* DNA into pMal-p2 was confirmed by the recognition of all *Bam*HI and *Sal*I digested DNA inserts by the Rx1 probe.

Expression and conformation of truncated recombinant proteins. The transformed *E. coli* strains BAR36A, BAR39, BAR66, BAR9739, BARL5, BAR6A and BAR100 were grown in LB media supplemented with 10 mM glucose and induced with 2 mM IPTG for expression of the truncated PspA protein fused with maltose binding protein. Transformed *E. coli* strains BC100 and BAR416, which expr ss PspA fragments fused to the OmpA leader sequence in

the pIN-III-ompA3 vector, were grown in minimal medium and induced with 2 mM IPTG for expression. Both vectors, pIN-III-ompA3 and pMal-p2, are vectors in which fusion proteins are exported to the periplasmic space. Therefore, an osmotic shock extract from the pMal-p2 containing bacteria was then run over an amylose column for purification and resolved by SDS-PAGE western blotting. The western blot of the protein extracts from BAR36A, BAR39, BAR66, BAR9739, BARL5, BAR6A and BAR100 were recognized by a rabbit polyclonal antibody made to strain BC100 PspA. The apparent M_r of full-length PspA from WU2 is 91.5 kD. The M_r of maltose binding protein is 42 kD and the expected M^r for the PspA portion of the fusion is 12 kD. All extracts exhibited molecular weights that ranged from 54 to 80 kD. This range of molecular weights can be attributed to the variability of *pspA* among different pneumococcal strains. An ELISA, with plates coated with the various cloned fragments quantitatively confirmed the reactivities that were observed in the western blots with all protein extracts.

Protection and cross-protection against fatal pneumococcal infection elicited by cloned PspA fragments. CBA/N mice were immunized with the truncated PspA fragment encoded by pBC100, which is composed of amino acids 192 to 588 of Rx1 PspA, and challenged with 13 different *S. pneumoniae* strains representing 7 different capsular types (Table 4). With all 13 strains, the immunization resulted in protection from death or an

extended time to death. With 10 of the strains the difference was statistically significant. With strains of capsular types 3, 6A, and 6B, all immunized mice were protected against death. Although there were fewer survivors in the case of capsular types 2, 4, and 5, the immunization with BC100 resulted in significant increases in times to death.

The BC100 immunization studies made it clear that epitopes C-terminal to residue 192 could elicit cross-protection. The BAR416 fragment, which includes amino acids 192-299, could elicit protection from fatal infection with a single challenge strain WU2. This Example shows the ability of BAR416 immunization to protect against the 6 strains that had been best protected against by immunization with BC100. Immunization with the BAR416 construct resulted in increased time to death for all 6 challenge strains examined (Table 5). BAR416 provided significant protection against death with WU2, A66, BG7322 and EF6796 pneumococci (capsular types 3, 3, 6B and 6A respectively). It also prolonged the lives of mice challenged with ATCC6303 and DBL6A pneumococci (capsular types 3 and 6A respectively). Serum from mice immunized with the BAR416 fragment yielded a geometric mean reciprocal anti-PspA ELISA titer to full-length Rx1 PspA of 750. Mice immunized with BC100 had geometric mean reciprocal titers of close to 2000, while non-immunized mice had anti-PspA titers of <10.

The above data indicates that the BAR416 fragment from Rx1 elicits adequate cross-reactive immunity to protect against diverse pneumococci and suggests that this region must be serologically conserved among PspAs. This hypothesis was confirmed by immunized with recombinant BAR416 homologous regions from the 7 different clones and then challenging with strain WU2 (Table 6). All 7 immunogens elicited significant protection. PspA fragments from capsular types 2 and 22 and the rough R36A strain elicited complete protection against death with all challenged mice. All of the other immunogens were able to extend the time to death of all the mice with the median days to death being 21 days or >21 days. Serum from mice immunized with the BAR416 homologous fragments had anti-PspA reciprocal titers that ranged from 260 to 75,800 with an average of 5700 while control animals immunized with only maltose binding protein had anti-PspA reciprocal titers of <10.

Antibody reactives. All of the above immunization studies attest to the cross-reactivity of epitopes encoded by amino acids from position 192-299. This region includes the C-terminal third of the α-helical region and the first amino acids of the proline rich region. Other evidence that epitopes within this region are cross-reactive among different PspAs comes form the cross-reactivity of a panel of nine MAbs all of which were made by immunization with Rx1 PspA. The epitopes of four of the antibodies in the panel r acted with epitopes mapping between

amino acids 192-260. The epitopes of the other five MAbs in the panel map between amino acids 1 and 115 (McDaniel, 1994). Each of these 9 MAbs were tested for its ability to react with 8 different PspAs in addition to Rx1. The 5 MAbs whose epitopes were located within the first 115 amino acids, reacted on average with only 1 other PspA. Three of the 5 in fact, did not react with any of the other 8 PspAs. In contrast the MAbs whose epitopes map between 192 and 260 amino acids each cross-reacted with an average of 4 of the 8 non-Rx1 PspAs, and all of them reacted with at least two non-Rx1 PspAs. Thus, based on this limited section of individual epitopes, it would appear that epitopes in the region from 192-260 amino acids are generally much more cross-reactive than epitopes in the region from 1-115 amino acids.

The BC100 fragment of Rx1 PspaA can elicit protection against the encapsulated type 3 strain WU2. Although the PspAs of the two strains can be distinguished serologically they are also cross-reactive (Crain, 1990). The earlier finding made it clear that epitopes cross-protective between Rx1 and WU2 PspAs exist. The importance of cross-reactions in the region C-terminal to residue 192 is demonstrated in this Example where 13 mouse virulent challenge strains have been used to elicit detectable protection against all of them. The first indication that epitopes C-terminal to position 192 might be able to elicit cross-prot ction came from our earlier study where we showed the

MAbs Xi64, XiR278, XiR1323, and XiR1325, whose epitopes mapped between amino acids 192 and 260 of strain Rx1 PspA, could protect against infection with WU2. Moreover, immunization with PspA fragments from 192-588 and 192-299 were able to elicit protection against infection against WU2. This Example shows that the BC100 Rx1 fragment (192-588) elicits significant protection against each of 13 different mouse virulent pneumococci, thereby firmly establishing the ability of epitopes C-terminal to position 192 to elicit a protective response. The observation that a fusion protein containing amino acids 192-299 fused C-terminal to maltose binding protein could also elicit cross-protection, permits the conclusion that epitopes in this 107 amino acid region of PspA are sufficient to elicit significant cross-protection against a number of different strains.

Evidence that a comparable region of other PspAs is also able to elicit cross-protection cam from the studies where sequences homologous to the 192-299 region of Rx1 PspA were made from 5 other PspAs. All 5 of these fragments elicited significant protection against challenge with strain WU2. These data provide some suggestion for serologic differences in cross-protection elicited by the 192-299 region.

Based on present evidence, without wishing to be bound by any one particular theory, it is submitted that the PspAs in strains D39, Rx1 and R36A are identical. All of the 9 mice immunized with the 192-299 fragments from R36A and D39 survived

challenge with WU2. Only LM100, one of the non-R36A/D39 PspAs, protected as high a percentage of mice from WU2. The difference in survival elicited by the R36A/D39 PspAs and the non-Rx1 related PspAs was statistically significant.

The data do indicate however, that all of the differences in protection against different strains are not due to differences in serologic cross-reactivity. BC100, which is made from Rx1, protected against death in 100% of the mice challenged with 7 different strains of *S. pneumonia*, but only delayed death with strain D39, which is thought to have the same PspA as strain Rx1. Thus, some of the differences in cross-protection are undoubtedly related to factors other than PspA cross-reactivity. Whether such factors are related to differences in virulence of the different strains in the hypersuceptible Xid mouse, or differences in requirements for epitopes N-terminal to amino acid 192, or difference in the role of PspA in different strains is not yet known.

These results suggest that a vaccine containing only the recombinant PspA fragments homologous with Rx1 amino acids 192-299 is effective against pneumococcal infection. Moreover, the results demonstrate that utility of PspA a.a. 192-299, a.a. 192-260 and DNA coding therefor, e.g. primers N192 or 588 (variants of LSM4 and LSM2) as useful for detecting the presence of pneumococciae by detecting presence of that which binds to the amino acid or to the DNA, or which is amplified by the DNA, e.g.,

by using that DNA as a hybridization probe, or as a PCR primer, or by using the amino acids in antibody-binding kits, assays or tests; and, the results demonstrate that a.a. 192-299 and a.a. 192-260 can be used to elicit antibodies for use in antibody-binding kits assays or tests.

Table 4 Protection of mice by immunization with BC100 from Rx1 PspA

Challenge Strain*	Capsule type	PspA type	BC100 Immunogen # alive / # dead	BC100 Immunogen % Survival	BC100 Immunogen Median days alive	Controls # alive / # dead	Controls % Survival	Controls Median days alive	P Value§
D39	2	25	0 / 5	0%	5	0 / 3	0%	2	0.02
WU2	3	1	4 / 0	100%	>21	0 / 3	0%	3	0.002
ATCC6303	3	7	5 / 0	100%	>21	0 / 5	0%	7	0.004
A66	3	13	4 / 0	100%	>21	0 / 3	0%	1	0.03
EF10197	3	18	5 / 0	100%	>21	0 / 3	0%	2	0.02
EF5668	4	12	1 / 3	25%	9	0 / 3	0%	4	N.S.
EF3296	4	20	1 / 3	25%	5	0 / 3	0%	3	N.S.
L81905	4	23	1 / 4	20%	4	0 / 6	0%	2	0.02
BG9739	4	26	0 / 4	0%	6.5	0 / 3	0%	2	N.S.
DBL5	5	33	0 / 5	0%	5	0 / 3	0%	2	0.02
BG7322	6	24	4 / 0	100%	>21	1 / 2	33.3%	6	0.03
EF6796	6A	1	4 / 0	100%	>21	0 / 3	0%	1	0.03
DBL6A	6A	19	5 / 0	100%	>21	0 / 3	0%	7	0.03

* Mice were challenged with approximately 10^3 CFU/mL of each strain
§ P values were based on comparison of days alive by a one-tailed Wilcoxon 2 sample-rank test

Table 5 Protection of mice by immunization with BAR416 from Rx1 PspA

Challenge Strain	Capsule type	PspA type	BAR416 Immunogen # alive / # dead	BAR416 Immunogen % Survival	BAR416 Immunogen Median days alive	Controls # alive / # dead	Controls % Survival	Controls Median days alive	P Value §
WU2	3	1	4 / 1	80%	>21	0 / 3	0%	1	0.002
ATCC6303	3	7	2 / 3	40%	13	1 / 4	20%	4	0.048
A66	3	13	5 / 0	100%	>21	0 / 5	0%	2	0.004
BG7322	6	24	3 / 2	60%	>21	0 / 4	0%	7	0.02
EF6796	6A	1	3 / 2	60%	>21	0 / 5	0%	5	0.004
DBL6A	6A	19	0 / 5	0%	7	0 / 5	0%	2	0.008

Note, mice were challenged with about 10^3 CFU of each strain
§ P values were based on comparison of days alive by a one-tailed Wilcoxon 2 sample-rank test

Table 6 Protection of mice against *S. pneumoniae* WU2 by immunization with BAR416 Analogs of 7 PspAs

Immunogen	Parent Strain	Capsule type	PspA type	# alive / total #	% Survival	Median days alive	P value* vs. MBP
BAR36A	R36A	-	25	4 / 4	100%	>21	0.002
BAR39	D39	2	25	5 / 5	100%	>21	0.0008
BAR66	A66	3	13	7 / 8	88%	>21	<0.0001
BAR9739	BG9739	4	26	5 / 8	63%	>21	0.0002
BARL5	DBL5	5	33	4 / 8	50 %	21	0.03
BAR6A	DBL6A	6A	19	3 / 5	60%	>21	0.05
BAR100	LM100	22	ND	5 / 5	100 %	>21	0.0008
MBP	-	-	-	0 / 8	0%	2	-

* P values were based on comparison of days alive by a one-tailed Wilcoxon 2 sample-rank test

Note, the PspA fragments used for immunization were cloned from products amplified with primers LSM4 and LSM6. In addition to the strains listed above, PCR reactions with LSM4 and LSM6 amplified products of the appropriate size from strains BG9163, WU2, L81905, EF6796, EF5668, BG7376, BG7322, and BG5-8A.

Table 7 Reactivity of MAbs with PspAs of Different Pneumococci

Donor of test PspA			MAb mapping to 1-115 amino acids					MAb mapping to 192-260 amino acids			
Strain	Capsule Type	PspA Type	Xi126 IgG2b	XiR1224 IgM	XiR1526 IgG2b	XiR35 IgG2a	XiR16 IgG2a	XiR1323 IgM	Xi64 IgM	XiR278 IgG1	XiR1325 IgG2a
Rx1	rough	25	++	++	++	++	++	++	++	++	++
ATCC101813	3	3	++	-	-	-	-	++	++	++	++
EF10197	3	18	-	-	-	-	-	-	-	++	+/-
BG9739	4	26	-	-	-	-	-	++	-	+	++
L81905	4	23	-	-	-	-	-	-	-	-	-
BG-5-8A	6A	0	+/-	+	-	-	-	+	-	+	-
BG9163	6B	21	-	-	-	-	-	-	-	+	-
LM100	22	N.D.	+/-	-	-	-	-	-	-	-	-
WU2	3	1	++	-	-	-	-	++	++	++	++

Note, immunoblot analysis was carried out with the nine MAbs from this study against a panel of nine different pneumococcal strains. Rx1 served as a positive control. The results are presented as ++ (strong reaction), + (weak, but clearly positive reaction), +/- (difficult to detect), and - (no reaction).The PspA of all strains gave a positive reaction with rabbit

antiserum against PspA. N.D. means not determined. Mapping of epitopes was to fragments of strain Rx1 PspA

EXAMPLE 3 - Isolation of PspA and Truncated Forms Thereof, and Immunization Thereby

PspA is attached to the pneumococcal surface through a choline binding site on PspA. This allows for successful procedures for the isolation of FL-PspA. PspA can be released from the surface of pneumococci by elution with 2 percent choline chloride (CC), or by growth in a chemically defined medium (CDM) containing 1.2 percent CC (CDM-CC) or medium in which the choline had been replaced by ethanolamine (CDM-ET). Since CDM-ET supernatants lack high concentrations of choline, the PspA released into them can be adsorbed to a choline (or choline analog) column and isolated by elution from the column with 2 percent choline chloride (CC).

This Example describes the ability to obtain PspA by these procedures, and the ability of PspA obtained by these procedures to elicit protection in mice against otherwise fatal pneumococcal sepsis. Native PspA from strains R36A, RX1, and WU2 was used because these strains have been used previously in studies of the ability of PspA to elicit protective immunity (see, e.g., Examples infra and supra). The first MAbs to PspA were made against PspA from strain R36A and the first cloned fragments of PspA and PspA mutants came from strain Rx1. Strain Rx1 was derived from strain R36A, which was in turn derived from the encapsulated type 2 strain, D39. PspAs from these three strains appears to be indentical based on serologic and molecular weight analysis. Molecular studies have shown no differences in the *pspA* genes of strains D39, Rx1, and R36A. The third strain that provided PspA in this Example is the mouse virulent capsular type 3 strain WU2. Its PspA is highly cross-reactive with that from R36A and Rx1, and immunization with Rx1 and D39 PspA can protect against otherwise fatal infections with strain WU2.

S. pneumoniae

Strains of *S. pneumoniae* used in this study have been described previously *(Table 8)*. Bacteria were grown in either Todd-Hewitt broth with 0.5 percent yeast extract (THY), or a chemically defined medium (CDM) described previously [32, 43]. Serial passage of stock cultures was avoided. Strains were maintained frozen in THY + 20 percent glycerol and cultured from a scraping of the frozen culture.

Recovery of PspA from pneumococci

PspA is not found in the medium of growing pneumococci unless they have reached stationary phase and autolysis has commenced [36]. To release PspA from pneumococci three procedures were used. In one approach were grow pneumococci in 100 ml of THY and collect the cells by centrifugation at mid-log phase. The pellet was washed three times in lactated Ringer's solution (Abbot Lab. North Chicago, IL), suspended in a small volume of 2 percent choline chloride in phosphate buffered saline (PBS) (pH 7.0), incubated for 10 minutes at room temperature, and centrifuged to remove the whole pneumococci. From immunoblots with anti-PspA MAb Xi126 [48] at serial dilutions of the original culture, the suspended pellet, and the supernatant, it was evident that this procedure released about half of the PspA originally present on the pneumococci. Analysis of silver stained polyacrylamide gels showed this supernatant to contain proteins in addition to PspA [36].

The CDM used in the remaining two procedures was modified from that of Van der Rijn [43]. For normal growth it contained 0.03% CC. To cause PspA to be released during bacterial growth, the pneumococci were grown in CDM containing 1.2 percent choline chloride (CDM-CC), or in CDM containing 0.03 percent ethanolamine and only 0.000,001 percent choline (CDM-ET). In media lacking a normal concentration of choline the F-antigen and C-polysaccharide contain phosphoethanolamine rather than phosphocholine [49]. In CDM-CC and CDM-ET, PspA is released from the pneumococcal surface because of its inability to bind to the cholines in the lipoteichoic acids [36]. In addition to releasing PspA from the pneumococcal surface, growth in CDM-CC or CDM-ET facilitates PspA isolation by its other effects on the cell wall. In these media pneumococci do not autolyse [49], thus permitting them to be grown into stationary phase to maximize the yield of PspA. In these media septation does not occur and the pneumococci grow in long chains [36, 49]. As the pneumococci reach stationary phase they die, cease making PspA, and rapidly settle out. Preliminary studies, using serial dilution dot blots to quantitate PspA, indicated that the production of PspA ceases at about the time the pneumococci begin to settle out, with the formation of visible strands of the condensed pneumococcal chains. When the pneumococci began to settle out, the medium was recovered by centrifugation at 2900 x g for 20 minutes, and filtered with a low protein-binding filter (.45μ Nalgene Tissue Culture Filter #158-0045).

For growth in CDM-CC or CDM-ET, the pneumococci were first adapted to the defined medium and then, in the case of CDM-ET, to very low choline concentrations. To do this, strains were first inoculated into 1 part of THY and 9 parts of CDM medium containing 0.03 percent choline and 0.03 percent ethanolamine. After two subsequent subcultures in CDM containing 0.03 percent choline and 0.03 percent ethanolamine (0.1 ml of culture + 0.9 ml of pre-warmed fresh medium), the culture was used to inoculate CDM with only 0.003 percent choline (and 0.03 percent ethanolamine). These steps was repeated until the strain would grow in CDM-ET containing 0.000,001 percent choline and 0.03 percent ethanolamine. It was critical that cultures be passed while in exponential growth phase (at about 10^7 CFU/ml). Even trace contamination of the medium by

exogenous choline resulted in the failure of the PspA to be released from the pneumococcal surface [36]. Thus, disposable plastic ware was used for the preparation of CDM-ET media and for growth of cultures. Once a strain was adapted to CDM-ET it was frozen in 80 percent CDM-ET and 20 percent glycerol at -80° C. When grown subsequently the strain was inoculated directly into the CDM-ET.

Isolation of native (full-length) PspA

PspA was isolated from the medium of cells grown in CDM-ET using choline-Sepharose prepared by conjugating choline to epoxy-activated Sepharose [50]. A separate column was used for media from different strains to avoid cross-contamination of their different PspAs. For isolation of PspA from clarified CDM-ET, we used a 0.6ml bed volume of choline-Sepharose. The column bed was about 0.5 cm high and 1.4 cm in diameter. The flow rate during loading and washing was approximately 3 ml/min. After loading 300 ml CDM-ET supernatant, the column was washed 10 times with 3 ml volumes of 50 mM Tris acetate buffer, pH 6.9 containing 0.25 M NaCl (TAB). The washed column was eluted with sequential 3 ml volumes of 2 percent CC in TAB. Protein eluted from the column was measured (Bio-Rad protein assay, Bio-Rad, Hercules, CA). The column was monitored by quantitative dot blot. The loading material, washes, and the eluted material were dot blotted (1µl) as undiluted, 1/4, 1/16, 1/64, 1/256, and 1/1024 on nitrocellulose. The membranes were then blocked with 1 percent BSA in PBS, incubated for 1 hr with PspA-specific MAbs Xi126 or XiR278, and developed with biotinylated goat-anti-mouse Ig, alkaline phosphatase conjugated streptavidin (Southern Biotechnology Associates Inc. Birmingham, AL), and nitrobluetetrazolium substrate with 5-bromo 4-chloro-3-indoyl phosphate *p*-toluidine salt (Fisher Scientific, Norcross GA) [17]. The purity of eluted PspA was assessed by silver-stained (silver stain kit, Bio Rad, Hercules, CA) SDS-PAGE gels run as described previously [32]. Immunoblots of SDS-PAGE gels were developed with MAbs Xi126 and XiR278 [17].

Isolation of 29 kDa PspA

The 29 kDa fragment comprising the N-terminal 260 amino acids of PspA was produced in DH1 *E. coli* from pJY4306 [31, 37]. An overnight culture of JY4306 was grown in 100 ml of Luria Broth (LB) containing 50µg/ml ampicillin. The culture was grown at 37° C in a shaker at 225 rpm. This culture was used to inoculate 6 one liter cultures that were grown under the same conditions. When the culture O.D. at 600 nm reached 0.7, 12 grams of cells, as a wet paste, were harvested at 4° C at 12,000 xg. The pellet was washed in 10 volumes of

25 mM Tris pH 7.7 at 0° C and suspended in 600 ml of 20% sucrose, 25 mM Tris pH 7.7 with 10 mM ethylenediamine tetraacetic acid (EDTA) for 10 minutes. The cells were pelleted by centrifugation (8000 xg) and rapidly suspended in 900 ml of 1 percent sucrose with 1 mM Pefabloc SC hydrochloride (Boehringer Mannheim Corp., Indianapolis, IN.) at 0° C. The suspension was pelleted at 8000 xg at 4° C for 15 minutes and the PspA-containing supernatant (periplasmic extract) [51] recovered. The recombinant PspA was precipitated from the periplasmic extract by 70 percent saturated ammonium sulfate overnight at 4° C. The precipitated material was collected by centrifugation at 12,000 xg at 4° C for 30 minutes. The precipitated protein was resuspended in 35 ml of 20 mM histidine 1 percent sucrose at pH 6.6 (HSB). Insoluble materials were removed at 1,000 xg at 4° C for 10 minutes. The clarified material was dialyzed versus HSB, passed through a 0.2µm filter and further purified on a 1 ml MonoQ HR 5/5 column (Pharmacia Biotech, Inc., Piscataway, N.J) equilibrated with HSB. The clarified material was loaded on the column at 1 ml/min, and the column was washed with 10 column volumes of HSB. The column was then eluted with a gradient change to 5 mM NaCl per minute at a flow rate of 1 ml/min. As detected by immuno blot with Xi126, SDS-PAGE and absorbance, PspA eluted as a single peak at approximately 0.27 to 0.30 M NaCl. By SDS-PAGE the material was approximately 90 percent pure. The yield from 6 liters of culture was 2 mg (Bio-Rad protein assay) of recombinant PspA.

Growth of pneumococci for challenge

Mice were challenged with log-phase pneumococci grown in THY. For challenge, the pneumococci were diluted directly into lactated Ringer's without prior washing or centrifugation. To inject the desired numbers of pneumococci, their concentration in lactated Ringer's solution was adjusted to an O.D. of about 0.2 at 420 nM (LKB Ultrospec III spectrophotometer). The number of pneumococci present was calculated at $5x10^8$ CFU per ml / O. D. and confirmed by colony counts (on blood agar) of serial dilutions of the inoculum.

Immunization, challenge, and bleeding of mice

CBA/CAHN/XID/J (CBA/N) and BALB/cByJ (BALB/c) mice were purchased from Jackson Laboratory Bar Harbor, ME. Mice were given two injections two weeks apart and challenged i.v. two weeks later. Injections without CFA were given intrapertioneally in a 0.1 ml of Ringers. Where indicated, the first injection was given in complete Freund's adjuvant (CFA) consisting of approximately a 1:1 emulsion of antigen solution and CFA oil (Difco, Detroit MI). Antigen in CFA was injected inguinally in 0.2 ml divided between the two hind legs. All mice were boosted i.p. without adjuvant. When mice were injected with media supernatants or 2 percent choline chloride eluates of whole bacteria, the amounts of material injected were expressed as the

volume of media from which the injected material was derived. For example, if the clarified medium from pneumococci grown in CDM-CC or CDM-ET was used for immunization without dilution or concentration, the dose was described as 100µl. If the material was first diluted 1/10, or concentrated 10 fold, the dose was referred to as 10 or 1000 µl respectively.

ELISA for antibodies to PspA

Specific modifications of previously reported ELISA conditions [17], are described. Microtitration plates (Nunc Maxisorp, P.G.C. Scientific, Gaithersburg MD.) were coated with undiluted supernatants of Rx1 and WG44.1 pneumococci grown in CDM-ET or 1 percent BSA in PBS. Mice were bled retro-orbitally (75 µl) in a heparanized capillary tube (Fisher Scientific, Fair Lawn, N.J.) The blood was immediately diluted in 0.5 ml of one percent bovine serum albumin in PBS. The dilution of the resultant sera was 1/15 based on an average hematocrit of 47 percent. The sera were diluted in 7 three fold dilution in microtitration wells starting at 1/45. Mab Xi126 was used as a positive control. The maximum reproducible O. D. observed with Xi126 was defined as "maximum O. D." The O. D. observed in the absence of immune sera or MAb was defined as "minimum O.D." Antibody titers were defined as the dilution that gives 33 percent of maximum O. D. The binding to the Rx1 CDM-ET coated plates was shown to be PspA-specific, since in no case did we observe ≥33 percent of maximum binding of immune sera or Xi126 on plates coated with WG44.1 CDM-ET or BSA.

Statistical analysis. Unless otherwise indicated *P* values refer to comparisons using the Wilcoxin two-sample rank test to compare the numbers of days to death in different groups. Mice alive at 21 days were assigned a value of 22 for the sake of calculation. *P* values of >0.05 have been regarded as not significant. Since we have never observed immunization with PspA or other antigens to make pneumococci more susceptible to infection the *P* values have been calculated as single tailed tests. To determine what the *P* value would have been if a two tailed test had been used the values given should be multiplied by two. In some cases *P* values were given for comparisons of alive versus dead. These were always calculated using the Fisher exact test. All statistical calculations were carried out on a Macintosh computer using InStat (San Diego, CA).

PspA is the major protection-eliciting component released from pneumococci grown in CDM-ET or CDM-CC, or released from conventionally grown pneumococci by elution with 2% CC.

PspA-containing preparations from pneumococci were able to protect mice from fatal sepsis following i.v. challenge with 3 x 10^3 (100 x LD_{50}) capsular type 3 *S. pneumoniae (Table*9 *)*. Comparable preparations from the strains unable to make PspA (WG44.1 and JY1119), or unable to make full length PspA (LM34 and JY2141) were unable to elicit protection. Regardless of the method of isolation the minimum protective dose was derived from pneumococci grown in from 10 - 30 µl of medium. We also observed 9 that supernatants of log phase pneumococci grown in normal THY or CDM media could not elicit protection (data not shown). This finding is consistent with earlier studies [36, 37] indicating the PspA is not normally released in quantity into the medium of growing pneumococci.

Isolated PspA can elicit protection against fatal infection

Although PspA was necessary for these preparations to elicit protection it was possible that it did not act alone. Mice were thus, immunized with purified FL-PspA to address this question.

Isolation of FL-PspA from CDM-ET growth medium. We isolated the FL-PspA from CDM-ET rather than from CDM-CC medium or a 2 percent choline chloride elution of live cells, because the high levels of choline present in the latter solutions prevents adsorption of the PspA to the choline residues on the choline-Sepharose column. PspA for immunization was isolated from strain R36A, as the strain is non-encapsulated and the isolated PspA could not be contaminated with capsular polysaccharide. As a control we have conducted mock isolations from WG44.1 since this strain has an inactivated *pspA* gene and produces no PspA. The results shown in *Table*10 are typical of isolations from 300 ml of CDM-ET medium from R36A grown pneumococci. We isolated 84 µg of PspA from 300 ml of medium, or about 280 µg/liter. Based on the dot blot results this appears to be about 75% of the PspA in the original medium, and that CDM-ET from R36A cultures contains about 400 µg/liter of PspA, or about 0.4 µg/ml.

No serologically detectable PspA was seen in the CDM-ET from WG44.1 cultures. More significantly there was undetectable protein recovered from the choline-Sepharose column after adsorption of CDM-ET from a WG44.1 culture, indicating that PspA is the only protein that could be isolated by this procedure. Moreover by silver stained SDS PAGE gel the PspA isolated from R36A appeared to be homogenous *(Figure.*3 *)*. Although autolysin can also be isolated on choline-Sepharose [20, 50], we did not expect it to be isolated by this procedure since autolysin is not released from pneumococci grown in choline deficient medium [36]. The

immunologic purity of the isolated PspA was emphasized by the fact that immunization with it did not elicit any antibodies detectable on plates coated with CDM-ET supernatants of WG44.1.

Loading more than 300 ml on the 0.6 ml bed volume column did not result in an increased yield, which suggested that the column capacity had been reached. However, increasing the depth of the choline-Sepharose bed to greater than 0.5 cm, decreased the amount of PspA eluted from the column, presumably because of non-specific trapping of aggregates in the column matrix. The elution buffer contains 50 mM Tris acetate 0.25 M NaCl and 2% choline chloride. Elution without added NaCl or with 1M NaCl resulted in lower yields. Elution with less than 1% CC also reduced yields.

Immunization of mice with purified R36A PspA. For immunization we used only the first 3 ml fraction of the R36A column. Mice were immunized with two injections of 1, 0.1, or 0.01 μg of R36A PspA, spaced two weeks apart. As controls, some mice were inoculated with a comparable dilutions of the first 3 ml fraction from the WG44.1 column. Purified FL-PspA elicited antibody to PspA at all doses regardless of whether CFA was used as an adjuvant *(Table 1)*. In the absence of CFA the highest levels of antibody were seen with the 1 μg dose of PspA. In the presence of CFA, however, the 0.1μg dose was as immunogenic as the 1 μg dose.

To test the ability of the different doses of PspA to elicit protection against challenge we infected the immunized mice with two capsular type 3 strains, WU2 and A66. Although both of these strains are able to kill highly susceptible CBA/N XID mice at challenge doses of less than 10^2, the A66 strain is several logs more virulent when BALB/c mice are used [47, 52]. The difference in virulence of A66 and WU2, was partially compensated for by challenging the immunized CBA/N mice with lower doses of strain A66 than WU2.

After immunization of CBA/N mice with 1 and 0.1μg doses of PspA we observed protection against WU2 challenge regardless of whether or not CFA was used as an adjuvant *(Table 4)*. At the lowest dose, 0.01 μg PspA, most of the mice immunized with PspA + CFA lived whereas most immunized with PspA alone did not; however, the difference was not statistically significant. When immunized mice were challenged with the more virulent strain A66 [47, 53], survivors were only observed among mice immunized with the 1 and 0.1 μg doses. There was slightly, more protection against fatal A66 infection among mice immunized with CFA than without, but the difference was not statistically significant. When the two sample rank test was used to analyze the time to death of mice infected with A66 we observed a statistically significant delay in the time to death in each immunized group as compared to the pooled controls.

The 29 kDa N-terminal fragment of PspA can elicit protection against infection when injected with CFA

We have compared the immunogenicity, with and without CFA, of an isolated 29 kDa fragment composed of the first 260 amino acids of PspA. Unlike the case with FL-PspA, adjuvant was required for the 29 kDa fragment to elicit a protective response. This was observed even though the immunizing doses of the 29 kDa antigen used were 10 and 30μg/mouse, or about 100 and 300 times the minimum dose of FL-PspA that can elicits protection in the absence of adjuvant.

Injection with CFA revealed the presence of additional protection eliciting antigen(s) in CDM-CC, and CDM-ET growth medium but not in the 2 percent choline chloride eluates of live cells

The observation that Freund's adjuvant could have such a major effect on the immunogenicity of the 29 kDa fragment *(Table12)*, prompted us to reexamine the immunogens described in *Table 2* to determine if immunization with adjuvant might enhance protection elicited by PspA-containing preparations or provide evidence for protection eliciting antigens in addition to PspA. By using CFA with the primary injection, the dose of PspA-containing growth medium (CDM-CC and CDM-ET) required to elicit protection was reduced from 10 - 30 μl *(Table9)* down to 1 to 3 μl *(Table13)*. When CFA was used as an adjuvant with CDM-CC and CDM-ET from PspA$^-$ strains WG44.1 and JY1119 we were able to elicit protective immune responses if material from ≥100μl or more of media were injected. Thus, although there were apparently some protection eliciting components other than PspA in CDC-CC and CDM-ET growth media, PspA remained the major protection eliciting component even in the presence of adjuvant.

One of the media used for injection was CDM-ET in which JY2141 had been grown. This medium elicited protection against WU2 challenge even when injected at doses as low as 1 μl. It should be noted that although this strain does not make full-length PspA, it secretes a truncated molecule comprising the first 115 amino acids of PspA into the growth medium. Thus, unlike CDM-ET from WG44.1 and JY1119, CDM-ET from JY2141 has the potential to elicit PspA-specific immunity. In contrast to these results, the material eluted from JY2141 with 2 percent CC was relatively non-immunogenic even when emulsified with CFA. This result is consistent with the fact that the 115 amino acid N-terminal PspA fragment of JY2141 is not surface attached [37], and would be expected to be washed away prior to the elution with 2 percent CC.

Extension of studies to BALB/c mice and i.p. challenge route

The studies above all involve i.v. challenge of CBA/N mice expressing with the XID genetic defect. The i.v. route, used in the present studies provides a relevant model for bacteremia and sepsis, but pneumococci have higher LD_{50}s when injected i.v. than i.p. CBA/N mice are hypersusceptible to pneumococcal infection because of the XID defect. This genetic defect prevents them from having circulating naturally occurring antibody to phosphocholine. The absence of these antibodies has been shown to make XID mice several logs

more susceptible to pneumococci than isogenic mice lacking the immune defect. From the data in *Table14* it is clear, however, that immunization with PspA can protect against infection in mice lacking the XID defect even when the challenge is by the i.p. route. Thus, there is no reason to suspect that the results presented are necessarily dependent on the use of the CBA/N XID mouse or the i.v. route.

PspA is highly immunogenic

These studies provide the first quantitative data on the amount of purified FL-PspA that is required to elicit protective immunity in mice. The isolated PspA for these studies was obtained by taking advantage of the fact that the C-terminal half of PspA binds to cell surface choline [36]. The isolated FL-PspA was found to be highly immunogenic in the mouse. Only two injections of 100 ng of PspA in the absence of adjuvant were required to elicit protection against otherwise fatal sepsis with greater than 100 LD_{50} of capsular type 3 *S. pneumoniae*. When the first injection was given with adjuvant, doses as small as 10 ng could elicit protective response. The potent immunogenicity of PspA, and the ability to isolate it on choline-Sehparose columns provides a demonstration for the possible use of PspA as a vaccine in humans.

A large body of published [17, 29, 37] as well as unpublished evidence indicates that the major protection eliciting epitopes of PspA are located in the α-helical (N-terminal) half of the molecule. From the present studies, it is clear that immunization with N-terminal fragments containing the first 115 or 260 of the 288 amino acid α-helical region are able to elicit protection when given with CFA. However, these fragment were not able to elicit protective responses without CFA. In the case of the both the 115 and 260 amino acid fragments, even immunization at 100 times the minimum dose that is immunogenic for FL-PspA failed to elicit a protective response. This result is consistent with previous results showing that a fragment composed of the N-terminal 245 amino acids [31, 37] could elicit protection against otherwise fatal pneumococcal infection of mice when the immunization was given with CFA[32]. In that study no immunization without CFA was attempted. Even though the C-terminal half of PspA may not contain major protection-eliciting epitopes it appears to contain sequence important in the immunogenicity of the molecule as a whole, since the full length molecule elicited much greater protection than the N-terminal fragments. The effect of the C terminal half on antigenicity may be in part that it doubles the size of the immunogen. Molecules containing the C-terminal half of PspA may also be especially immunogenic because they exhibit more extensive aggregation than is seen with fragments expressing only the α-helical region [38]. Protein aggregates are known to generally be more antigenic and less tolerogenic than individual free molecules [54].

PspA is the major protection eliciting component of our pneumococcal extracts

Evidence that PspA is the major protection eliciting component of the CDM-ET, CDM-CC growth media and the two percent CC eluates was dependent on the use of mutant pneumococci that lacked the ability to produce FL-PspA. More than one *pspA* mutant strain was used to insure that the failure to elicit protection in the absence of FL-PspA was not a spurious result of non-PspA mutation blocking the production of some other antigen. Strains WG44.1 and JY1119 contain identical deletions that include the 5' end of the *pspA* genes and extend about 3 kb upstream of *pspA*[37]. WG44.1 is a mutant of the non-encapsulated strain Rx1 and JY1119 was made by transforming capsular type 3 strain WU2 with the WG44.1 *pspA* mutation. In no case were preparations from WG44.1 and JY1119 as efficient at eliciting protection as those from the PspA$^+$ strains. To rule out the possibility that protection elicited by preparations from the PspA$^+$ strains was elicited by some non-PspA molecule also encoded by a 3 kb deletion linked to the mutant *pspA* genes of WG44.1 and JY1119, we also used strains JY2141 and LM34 [26, 37]. In these strains the Rx1 *pspA* gene has been insertionally inactivated causing the production of N-terminal fragments of 115 and 245 amino acids respectively. These strains have no other known mutations. Although Rx1 and R36A are closely related non-encapsulated strains, some of the studies included Rx1 as the PspA$^+$ control since it is the isogenic partner to WG44.1, LM34, and JY2141. The N-terminal fragments produced by JY2141 and LM34 lack the surface anchor and are secreted into the medium [36]. Two percent CC eluates of JY2141 were non-protection eliciting even in the presence of adjuvant. In the absence of adjuvant, CDM-ET from JY2141 was not protection-eliciting. LM34 was tested without CFA in only 3 mice, but gave results consistent with those obtained with JY2141.

Anticapsular antibodies are known to be protective against pneumococcal infection [5, 19]. However, in these studies it is unlikely that they account for any of the protection we attributed to PspA. Our challenge strain bore the type 3 capsular polysaccharide and our primary source of PspA was strain R36A, which is a spontaneous non-encapsulated mutant of a capsular type 2 strain [39, 41]. The R36A strain has been recently demonstrated to lack detectable type 3 capsule on the surface or in its cytoplasm [55]. Furthermore, the CBA/N mice used in most of the studies are unable to make antibody responses to capsular type 3 polysaccharide [56].

Non-PspA protection eliciting components

The observation that CDM-CC and CDM-ET supernatants of WG44.1 could elicit protection when injected in large amounts with adjuvant, suggested that these supernatants contained at least trace amounts of non-PspA protection eliciting molecules. In the case of preparations containing PspA eluted from the surface of live washed pneumococci with 2 percent CC, there was no evidence for any protection eliciting components other than PspA, presumably because the protection-eliciting non-PspA proteins released into the media were removed by the previous washing step. The identity of the protection eliciting molecules in the WG44.1

supernatant are unknown. In this regard, it is of interest that unlike R36A, strain Rx1 has been shown to contain a very small amount of cytoplasmic type 3 polysaccharide (but totally lacks surface type 3 polysaccharide[55]). This difference from Rx1 apparently came about through genetic manipulations in the construction of Rx1 from R36A [39, 41]. Thus, preparations made from Rx1 or from its daughter strains WG44.1, LM34, or JY2141 could potentially contain small amounts of capsular polysaccharide. For a number of reasons however, it seems very unlikely that the non-PspA protection-eliciting material identified in these studies was type 3 capsular polysaccharide (expressed by the WU2 challenge strain: 1) growth of these strains was either in CDM-CC or CDM-ET, each of which prevent autolysin activity and lysis [57] that would be required to release the small amount of type 3 polysaccharide from the cytoplasm of the Rx1 family of strains; 2) CBA/N mice made protective responses to the non-PspA antigens, but express the XID immune response deficiency which permits responses to proteins, but blocks antibody to most polysaccharides [46], including type 3 capsular polysaccharide [56]; and 3) immunogenicity of the non-PspA component required CFA, an adjuvant known to stimulate T-dependent (protein) rather than T-independent (polysaccharide) antibody responses.

A number of non-PspA protection eliciting pneumococcal proteins have been identified: pneumolysin, autolysin, neuraminidase, and PsaA which are 52, 36.5, 107 and 37 kDa respectively [21, 58, 59, 60]. The non-PspA protection eliciting components reported here could be composed of a mixture of these and/or other non-identified proteins. Attempts to identify lambda clones producing non-PspA protection eliciting proteins as efficacious as PspA have not been successful [25].

Isolation of PspA

The protective capacity of the CDM-CC, CDM-ET and material eluted from live cells with 2% CC were similar in terms of the volume of the original culture from which the injected dose was derived. The major advantage of eluting the PspA from the surface of pneumococci with 2 percent CC is that the pneumococci may be grown in any standard growth medium, and do not have to be first adapted to a defined medium. Moreover, concentration of PspA can be accomplished by centrifugation of the pneumococci prior to the elution of the PspA. An advantage of using either CDM-CC and CDM-ET media was that these media prevented lysis and pneumococci could be grown into stationary phase without contaminating the preparations with cytoplasmic contents and membrane and wall components. A particular advantage of CDM-ET growth medium is that since it lacks high concentrations of choline the PspA contained in it can be adsorbed directly to a choline-Sepharose column for affinity purification.

One liter of CDM-ET growth medium contains about 400 µg of PspA, and we were able to isolate about 3/4 of it to very high purity. At 0.1 µg/dose, a liter of CDM-ET contains enough PspA to immunize about 4,000

mice; or possibly 40 - 400 humans. Our present batch size for a single column run is only 300 ml of CDM-ET. This could presumably be increased by increasing the amount of the adsorbent surface by increasing the diameter of the column. Using our present running buffer we have found that a choline-Sepharose resin depth of 0.5 cm was optimal; increases beyond 0.5 cm caused the overall yield to decrease rather than increase, even in the presence of larger loading volumes of R36A CDM-ET

Table 8 Pneumococcal Strains

Strain	Capsule type	PspA expressed	Parent strain	Construction technique	References
D39	2	full length	–	clinical isolate	26, 44
R36A	non-encapsulated	full length	D39	non-encapsulated mutant	23, 44, 45
Rx1	non-encapsulated	full length	R36A	derived from R36A	26, 39, 41
WG44.1	non-encapsulated	none	Rx1	aberrant insertion inactivation with pKSD300	26, 37
LM34	non-encapsulated	aa 1- 245 of Rx1[a]	Rx1	insertional inactivation with pKSD300	26, 37, 42
JY2141	non-encapsulated	aa 1- 115 of Rx1[a]	Rx1	insertional inactivation with pJY4208	37
WU2	3	full length	–	clinical isolate	25, 46
JY1119	3	none	WU2	transformation with WG44.1 DNA	37
A66	3	full length	--	clinical isolate	44, 47

[a] LM34 and LY2141 express fragments containing the first 245 and first 115 amino acids of Rx1 PspA respectively.

Table 9 PspA is the major protection-eliciting component in antigen preparations made by three different methods

Preparation	Strain (PspA status)	Dose as volume of media in µl[a]	Median Days Alive	Alive: Dead	*P* versus controls[b]
2% CC eluate from live cells	R36A ($PspA^+$)	1000	>21	2 : 0	
		200	>21	2 : 0	
		20	>21	2 : 0	
		2	1.5	0 : 2	
		all R36A	>21	6 : 2	0.03
	JY2141 (aa 1 - 115)	1000	3, >21	1 : 1	
		200	1	0 : 2	
		20	1	0 : 2	
CDM-CC clarified medium	Rx1 ($PspA^+$)	100	>21	9 : 0	<0.0001
		30	>21	2 : 1	
		10	2	1 : 2	
		3	2	0 : 3	
		ALL	2,>21	12 : 6	0.0004
	LM34	100	2, 2, >21	1 : 2	
	WG44.1 ($PspA^-$)	100	2	0 : 9	
		30	2	0 : 3	
		10	2	0 : 3	
		4	2	0 : 3	
	WU2 ($PspA^+$)	1000	>21	3 : 0	0.05
		100	>21	1 : 0	
		ALL	>21	4 : 0	0.03
	JY1119 ($PspA^-$)	1000	4	0 : 3	
	CDM-CC	100	2	0 : 2	
CDM-ET clarified medium	R36A ($PspA^+$)	100	>21	8 : 0	<0.0001
		10	3, >21	5 : 5	0.004
		1	1.5	3 : 5	
		0.1	2	0 : 2	
		ALL	>21	16 : 12	0.006
	JY2141 (aa 1 - 115)	100	1.5	0 : 2	
		10	1.5	0 : 2	
	WG44.1 ($PspA^-$)	100	3	0 : 2	
		10	1.5	0 : 2	
None	–		2	0 : 14	–

[a] Antigen dose is given as the volume of growth media from which the 0.1 ml of injected material was derived. Each mouse was injected twice i.p. with the indicated dose diluted as necessary in lactated Ringer's injection solution.

[b] Controls used for statistical comparisons: 2% CC, all JY2141; CDM-CC Rx1, all WG44.1; CDM-CC WU2, JY1119; CDM-ET, all WG44.1 + all JY2141.

Table10 Isolation of PspA from 300ml of CDM-ET media after the growth of R36A or WG44.1 pneumococci[a]

fraction	R36A				WG44.1		
	µg protein/ml	total µg protein[b]	max. reciprocal dot blot[c]	total dot blot units[b, d]	µg protein per/ml	total µg protein[b]	max. reciprocal dot blot[c]
growth media	13.3	3,990	4	1200	13.7	4,110	<1
fall-through	13.6	4,080	1	300	13.5	4,050	<1
1st wash			<1				<1
10th wash			<1				<1
elution #1	26	78	256	770	<1	–	<1
elution #2	2	6	16	48	<1	–	<1
elution #3	<1	–	4	12	<1	–	<1
total eluted		84		830		–	<1

[a] The columns were loaded with 300 ml of clarified CDM-ET medium after the growth of R36A or WG44.1. The column was washed with 10 sequential 3 ml fractions of TBA. Elution was with TBA plus 2 percent CC.

[b] Total µg protein or total dot blot units reflect the total protein in the 300 ml of the loading material or the 3 ml size of the eluted fractions.

[c] MAb XiR278 was used in the immunoblots to detect PspA in dot blots.

[d] Dot blot units were calculated as the reciprocal dot blot titer times the volume in ml.

Table 11 Purified full-length PspA is able to elicit protection against fatal sepsis in mice.

Antigen	Dose[a]	Adjuvant or Diluent	Anti- PspA titer[b] (Log mean ± S.E.)	Challenge with $10^{5.1}$ WU2			Challenge with $10^{4.2}$ A66		
				Alive : Dead	Median Days Alive	*P* vs. pooled control[c]	Alive : Dead	Median Days Alive	*P* vs. pooled controls[c]
R36A (PspA+)	1 µg	Ringer's	3.3 ± 0.2	5 : 0	>21	0.015	2 : 3	4	0.002
	0.1	Ringer's	2.6 ± 0.2	4 : 0	>21	0.041	1 : 4	4	0.0032
	0.01	Ringer's	2.7 ± 0.2	1 : 4	4	n.s.	0 : 5	3	0.0058
	1 µg	CFA	3.5 ± 0.2	5 : 0	>21	0.027	3 : 2	>21	0.0012
	0.1	CFA	3.6 ± 0.1	5 : 0	>21	0.013	2 : 3	4	0.0012
	0.01	CFA	3.1 ± 0.2	4 : 1	>21	0.015	0 : 5	3	0.0058
WG44.1 (PspA-)	3600 µl	Ringer's	<1.6	n.d.	n.d.		1 : 4	3	n.s.
	360	Ringer's	<1.6	n.d.	n.d.		0 : 5	2	n.s.
	36	Ringer's	<1.6	n.d.	n.d.		0 : 5	2	n.s.
	3600 µl	CFA	<1.6	n.d.	n.d.		0 : 5	2	n.s.
	360	CFA	<1.6	n.d.	n.d.		1 : 4	2	n.s.
	36	CFA	<1.6	n.d.	n.d.		0 : 5	2	n.s.
saline	–	CFA	<1.6	1 : 5	4	--	n.d.	n.d.	–
pooled controls			<1.6	1 : 5	4		2 : 28	2	–

[a] For comparison with the data in *Table 2*, it should be noted that the 1, 0.1, and 0.01 µg doses were derived from 3600, 360, and 36 µl of R36A growth media. Equivalent dilutions of the PspA- eluate from strain WG44.1 were injected as controls. The amount of the WG44.1 preparations injected is listed as 3600, 360, and 36 µl and corresponds to the volume original growth medium from which the doses of WG44.1 was prepared.

[b] Antibody values were expressed as reciprocal ELISA titer.

[c] *P* values calculated by the Wilcoxon two sample rank test. By Kruskal-Wallis nonparametric ANOVA for the WU2 challenge was significant at $P=0.01$, for A66 significance was at $P<0.0001$.

Table 12 The 29 kDa N-terminal fragment of Rx1 PspA must be injected with adjuvant to elicit protection against WU2[a]

µg 29 kDa PspA	Adjuvant or diluent	Median Days Alive	Alive : Dead	*P* versus none[b]
30	CFA	>21	3 : 0	0.0006
3	CFA	>21	3 : 0	
30	Ringer's	2	0 : 3	
3	Ringer's	2	1 : 2	
none	CFA	2	0 : 7	
none	Ringer's	2	0 : 7	

[a]The 29 kDa fragment comprises the first 260 amino acids of PspA.

[b]For the calculation of *P* values the 30µg and 3 µg data were pooled; mice immunized with PspA + CFA were compared to CFA controls; mice immunized with PspA + Ringer's were compared to controls immunized with Ringer's. Only the statistically significant *P* values are shown. The calculated *P* value of PspA + CFA versus CFA alone, was 0.0006 by both the Wilcoxon two sample rank test and the Fisher exact test.

Table13 PspA is not the only protection eliciting molecule released from pneumococci by interference with binding to choline on the surface of pneumococci

Preparation	Strain (PspA status)	Dose (as volume in µl)	Median Day Alive	Alive: Dead	*P* values[a]
					P vs. all JY2141
2% CC	R36A	1000	>21	2 : 0	
eluate from	($PspA^+$)	200	>21	5 : 0	0.02
live cells		20	>21	5 : 0	0.02
		2	>21	5 : 0	0.02
		all R36A	>21	17 : 0	0.001
	JY2141	1000	>21	2 : 0	
	(aa 1 - 115)	200	1	0 : 2	
		20	1	0 : 2	
		2	1	0 : 2	
		all JY2141	1	2 : 6	
					P versus pooled cont.
CDM-CC	Rx1	1000	>21	3 : 0	0.002
clarified	($PspA^+$)	100	>21	3 : 0	0.002
medium					
+	WU2	1000	>21	3 : 0	0.002
CFA	($PspA^+$)	100	>21	3 : 0	0.002
		3	>21	3 : 0	0.002
	WG44.1	1000	>21	5 : 1	<0.0001
	($PspA^-$)	100	2.5	2 : 4	0.002
	JY1119 ($PspA^-$)	1000	>21	3 : 0	0.002
		100	>21	3 : 0	0.002
CDM-ET	R36A	1000	>21	3 : 1	0.004
clarified	($PspA^+$)	10	>21	4 : 0	0.004
medium		1	>21	3 : 1	0.004
+ CFA		0.2	2	0 : 4	
	JY2141	10	>21	2 : 0	
	(aa 1 - 115)	1	>21	2 : 0	
	all JY2141	-	>21	4 : 0	0.004
	WG44.1	100	>21	2 : 0	
	($PspA^-$)	10	2	0 : 2	
CDM-ET only	+ CFA		2	0 : 9	
None	none		1.5	0 : 4	
Pooled Controls[b]			2	0 :13	

[a] In cases where there were not statistically significant results no *P* value was shown.

[b] "Pooled Controls" refers to "CDM-ET only" Data and "None" data.

Table 14 Immunization of BALB/c mice with isolated PspA elicits protection against WU2 *S. pneumoniae*

Antigen		Adjuvant	Challenge		Days to	*P* vs. controls
Source	Dose[a]	or diluent	Log CFU	Route	Death	TSR/FE[b]
R36A (PspA$^+$)	1μg	CFA	4	i.p.	2, >21, >21, >21	0.06/0.03
WG44.1 (PspA$^-$)	100μl	CFA	4	i.p.	2, 3	
None	–	CFA	4	i.p.	2, 2, 2, 4	
R36A (PspA$^+$)	1μg	none	6	i.v.	2, >21, >21, >21	0.06/0.03
WG44.1 (PspA$^-$)	100μl	none	6	i.v.	5, 7	
none	–	none	6	i.v.	2, 2, 2, 3	
Pooled i.v and i.p. results				i.v. or i.p.		0.008/0.0007

[a] The 1μg dose of R36A PspA was isolated from 100μl of CDM-ET medium. As a control mice were injected with an corresponding volume of choline-column effluent from a mock isolation of PspA from the PspA$^-$ strain WG44.1. The dose of WG44.1 material is expressed as 100 μl since this is the volume CDM-ET from which the injected column effluent was derived.

[b] *P* values calculated by Wilcoxon two-sample rank test, TSR, or Fisher exact, FE versus pooled controls for each group. "Pooled controls" include data obtained with by injection of "WG44.1" and "none". The i.p. and i.v. studies gave comparable results. When the data from the two studies were pooled the *P* values by both tests were ≤0.008. In cases where there were not statistically significant results no *P* value was shown.

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EXAMPLE 4 - Evidence For Simultaneous Expression of Two PspAs

From Southern blot analysis there has been an issue as to whether most isolates of *S. pneumoniae* has two DNA sequences that hybridize with both 5′ and 3′ halves of Rx1 pspA, or whether this is an artifact of Southern blot (9). When bacterial lysates have been examined by Western blot, the results have always been consistent with the production of a single PspA by each isolate (1,10). This Examiner provides evidence for the first time that two PspAs of different apparent molecular weights and different serotypes can be simultaneously expressed by the same isolate.

Different PspAs frequently share cross-reactive epitopes, and an immune serum to one PspA was able to recognize PspAs on all pneumococci (1). In spite of these similarities, PspAs of different strains can generally be distinguished by their molecular weights and by their reactivity with a panel of PspA-specific monoclonal antibodies (MAbs) (1).

A serotyping system for PspA has been developed which uses a panel of seven MAbs. PspA serotypes are designated based on the pattern of positive or negative reactivity in immunoblots with this panel of MAbs. Among a panel of 57 independent isolates of 9 capsular groups/types, we observed 31 PspA serotypes (1). The large diversity of PspA was substantiated in a subsequent study of 51 capsular serotype 6B isolates from Alaska, provided by Alan Parkinson at the Arctic Investigations Laboratory of the Centers for Disease Control and Prevention.

Among these 51 capsular type 6B isolates we observed 22 different PspAs based on PspA serotype and molecular weight variations of Pspa (4, 11).

While most pneumococcal strains appear to have two DNA sequences homologous with both the 5′ and 3′ halves of pspA (9), site-specific truncation mutations of Rx1 have revealed that one these, pspA, encodes PspA (8, 9). The other sequence has been provisionally designated as the pspA-like sequence. At present whether the pspA-like sequence makes a gene product is unknown. Evidence that the pspA and pspA-like genes are homologous but distinct groups of alleles comes from Southern blot analysis at high stringencies (9). Additional evidence that pspA and the pspA-like loci are distinct comes from studies using PCR primers that permit amplification of a single product approximately 2Kb in size from 70% of pneumococci. For the remaining 30% of pneumococci no amplification was observed with the primers used (12).

Evidence for two PspAs:

When the strains of MC25-28 were examined with the panel of seven MAbs specific for different PspA epitopes, all four demonstrated the same patterns of reactivity (Fig. 4). The MAbs XiR278 and 2A4 detected a PspA molecule with an apparent molecular weight of 190 KDa in each isolate. In accordance with our previous PspA serotyping system, the 190 KDa molecule was designated as PspA type 6 because of its reactivity with XiR278

and 2A4, but none of the five other MAbs in the typing system. Each isolate also produced a second PspA molecule with an apparent molecular weight 82 KDa. The 82 KDs PspA is each isolate was detected only with the MAb 7D2 and was designated as type 34. No reactivity was detected with MAbs Xi126, Xi64, 1A4, or SR4W4. The fact that all four capsular 6B strains exhibit two PspAs, based on both molecular weights and PspA serotypes, suggested that they might be members of the same clone.

Simultaneous production of both PspAs:

Results from the colony immunobloting showed that both PspAs were present simultaneously in each colony of these isolates when grown *in vitro*. All colonies on each plate of the original culture, as well as all of the progeny colonies from a single colony, reacted with MAbs XiR278, 2A4, and 7D2 as previously described.

Number of *pspA* genes:

One explanation for the second PspA molecule was that these strains contained an extra *pspA* gene. Since most strains contain a *pspA* gene and a *pspA*-like gene (9) we expected that if an extra gene were present we might observe at least three *pspA* homologous loci in isolates MC25-28. In *Hind* III digests of MC25-28 each strain revealed a 7.7 and 3.6 Kb band when probed with plSM*pspA*13/2 (Figure 5A). In comparison, when Rx1 DNA was digested with *Hind* III and hybridized with plSM*pspA*13.2, homologous sequences were detected on 9.1 and 4.2 Kb fragments as

expected from previous studies (9) (Figure 5A). Results consistent with only two *pspA*-homologous genes in MC25-28 were also obtained with digestion using four additional enzymes (Table 15).

In previous studies it has been reported that probes for the 5′ half of *pspA* (encoding the a-helical half of the protein) bind the *pspA*-like sequence of most strains only at a stringency of around 90% (9). With chromosomal digests of MC25-28 we observed that the 5′ Rx1 probe of pLSM*pspA*12/6 bound both *pspA* homologous bands at a stringency of greater than 95 percent. The same probe bound only the *pspA* containing fragment Rx1 at a stringency above 95 percent (Figure 5B).

Further characterization of the *pspA* gene was done by RFLP analysis of PCR amplified *pspA* from each strain. Since previous studies indicated that individual strains yielded only one product, and since the amplification is carried out with primers based on a known *pspA* sequence, it seems likely that in each case the amplified products represent the *pspA* rather than the *pspA*-like gene (12). When MC25-28 were subjected to this procedure, an amplified *pspA* product of 2.1 Kb was produced in each case. When digested with *Hha* 1 digest the sum of the fragments obtained with each enzyme was approximately equal to the size of the 2.1 Kb amplified product (Figure 6). These results suggest that the 2.1 Kb amplified DNA represents the amplified product of only a single DNA sequence. Rx1, by

comparison, produced an amplified product of 2.0 Kb and five fragments of 0.76, 0.468, 0390, 0.349 and 0.120, when digested with *Hha* 1 as expected from its known *pspA* sequence (5).

The four isolates examined in this Example are the first in which two PspAs have unambiguously been observed. The interpretation that two PspAs are simultaneously expressed by a single pneumococcal isolate is based on the observation that bands of different molecular weights were detected by different MAbs to PspA. Isolates used in this study were from a group originally selected for study by Brian Spratt because of their resistance to penicillin. It is very likely that all four of the isolates making two PspAs are related since they share PspA serotypes, amplified *pspA* RFLPs, chromosomal *pspA* RFLPs, capsule type, and resistance to penicillin.

The interpretation of studies presented her, showing the existence of two PspAs in the four strains MC25-28, must be set in the context of what is know about the serology PspA as detected by Western blots. PspAs of different strains have been shown previously to exhibit apparent molecular weight sizes ranging from 60 to 200 KDa as detected by Western blots (1, 10). At least part of this difference in size is attributable to secondary structure (8, 10). Even for the PspA of a single isolate, band of several sizes are generally observed. Mutation and immunochemistry studies have demonstrated, however, that all of the different sized PspA band from Rx1 are made by a single

gene capable of encoding a 69 KDa protein (1). The heterogeneity of band size on Western blots of PspA made by a single strain appears to be due to both degradation and polymerization (13).

PspA was originally defined by reciprocal absorption studies demonstrating that a panel of MAbs to Rx1 surface proteins each reacted with some protein (14) and later by studies using Rx1 and WU2 derivatives expressing various truncated forms of PspA (8). In both cases it was clear that each of our MAbs to the PspA of a given strain reacted with the same protein. Such detailed studies have not been done with each of the several hundred human isolates. It is possible that with some isolates, reactivity of the MAbs with two PspAs may have gone unnoticed. This could have happened if all reactive antibodies detected both PspAs of the same isolate, or if the most prominent migration bands from each of the two PspAs co-migrated. With isolates MC25-28 the observation of two PspAs was possible because clearly distinguishable bands of different molecular weights reacted preferentially with different MAbs.

Applicants favor the interpretation that isolates MC25-28 each make two PspAs, because an alternative possibility, namely, that the 190 KDa PspA detected by MAbs XiR278 and 2A4 might be a dimer of the 84 KDa monomer detected by MAb 7D2, if the epitopes recognized by the different MAbs were dependent on ither the dimeric or monomeric status of the protein, seems unlikely since whenever MAbs react with the PspA of a strain,

they usually detect both the monomeric and the dimeric forms. No other isolates have been observed where some MAbs detected only the apparent dimeric form of PspA while others detected only the monomeric form.

There could be several possible explanations for the failure to observe two PspAs produced by most strains. 1) All pneumococci might make two *pspA*s in culture, but MAbs generally recognize only one of them (perhaps in this isolate there has been a recombination between *pspa* DNA and the *pspA*-like locus, thus allowing that locus to make a product detected by MAb to PspA). 2) All pneumococci can have two *pspAs* but the expression of one of them generally does not occur under *in vitro* growth conditions. 3) The *pspA*-like locus is normally a nonfunctional pseudogene sequence that for an unexplained reason has become functional in these isolates.

It seems unlikely that the expression of only a single PspA by most strains is the result of a phase shift that permits the expression of only the *pspA* or *pspA*-like gene at any one time, since many of the strains examined repeatedly and consistently product the same PspA (10, and unpublished data). In the case of strains MC25-28, the appearance of two PspAs is apparently not the result of a phase switch, since individual colonies produced both the type 6 and the type 34 PspAs.

Presumably in these four strains, the second PspA protein is produced by the *pspA*-like DNA sequence. At high

stringency, the probe comprising the coding region of the a-helical half of PspA recognized both *pspA* homologous sequences of MC25-28 but not the *pspA*-like sequence of Rx1. This finding indicates that the *pspA*-like sequence of MC25-28 is more similar to the Rx1 *pspA* sequence than is the Rx1 *pspA*-like sequence. If the *pspA*-like sequence of these strains is more similar to *pspA* than most *pspA*-like sequences, it could explain why we were able to see the products of *pspA*-like genes of these strains with our MAbs. The finding of two families of PspAs made *in vivo* by pneumococci, allows for use of the second PspA in compositions, as well as the use of DNA primers or probes for the second gene for more conclusive detecting, determining or isolating of pneumococci.

Isolates and Bacterial Cell Culture:

Pneumococcal isolates described in these studies were cultured from patients in Barcelona, Spain (one adult at Bellvitge Hospital, and three children at San Juan de Dios) between 1986 and 1988 (Table 2). These penicillin resistant pneumococci originally in the collection of Dr. Brian Spratt were shared with applicants by Dr. Alexander Tomasz at the Rockefeller Institute. Rx1 is a rough pneumococcus used in previous studies, and it is the first isolate in which *pspA* was sequenced (3, 8, 15). Bacteria were grown in Todd-Hewitt broth with 0.5% yeast extract or on blood agar plates overnight in a candle jar. Capsular serotype was confirmed by cell agglutination using Danish

antisera (Statens Seruminstitut, Copenhagen, Denmark) as previously described (13). The isolates were subsequently typed as 6B by Quellung reaction, utilizing rabbit antisera against 6A or 6B capsule antigen prepared by Dr. Barry Gray (16).

Bacterial lysates:

Cell lysates were prepared by incubating the bacterial cell pellet with 0.1% sodium deoxycholate, 0.01% sodium dedecylsulfate (SDS), and 0.15 M sodium citrate, and then diluting the lysate in 0.5M Tris hydrochloride (pH 6.8) as previously described (1). Total pneumococcal protein in the lysates was quantitated by the bicinchonic acid method (BCA Protein Assay Reagent; Pierce Chemical Company, Rockford, IL) (17).

PspA serotyping:

Serotyping of PspA was performed according to previously published methods (1). Briefly, pneumococcal cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and developed as Western blots using a panel of seven MAbs to PspA. PspA serotypes were assigned based on the particular combination of MAbs with which each PspA was reactive.

Colony Immunoblotting:

A ten ml. tube of Todd-Hewitt broth with 0.5% yeast extract was inoculated with overnight growth of MC23 from a blood agar plate. The isolate was allowed to grow to a concentration of 10^7 cells/ml as determined by an O.D. of 0.07 at 590nm. MC23

was serially diluted and spread-plated on blood agar plates to give approximately 100 cells per plate. The plates were allowed to grow overnight in a candle jar, and a single block agar plate with well-defined colonies was selected. Four nitrocellulose membranes were consecutively placed on the plate. Each membrane was lightly weighted and left in place for 5 minutes. In order to investigate the possibility of phase-variation between the two proteins detected on Western blots a single colony was picked from the plate, resuspended in ringers, and spread-plated onto a blood agar plate. The membranes were developed as Western blots according to PspA serotyping methods (1).

Chromosomal DNA Preparation:

Pneumococcal chromosomal DNA was prepared as previously described (15). The cells were harvested, washed, lysed, and digested with 0.5% (wt/vol) SDS and 100μg/ml proteinase K at 37°C for 1 hour (18, 19). The cell wall debris, proteins, and polysccharides were complexed with 1% hexadecyl trimethyl ammonium bromide (CTAB) and 0.7M sodium chloride at 65°C for 20 minutes, then extracted with chloroform/isoamyl alcohol. DNA was precipitated with 0.6 volumes isopropanol, washed, and resuspended in 10mM Tris-HCL, 1mM EDTA, pH 8.0 (20). DNA concentration was determined by spectrophotometric analysis at 260nm.

Probe preparation:

5′ and 3′ oligonucleotide primers homologous with nucleotides 1 to 26 and 1967 to 1990 of Rx1 *pspA* (LSM 13 and

LSM2, respectively) were used to amplify the full length *pspA* and construct probe LSM*pspA*13/2 from Rx1 genomic DNA (21). 5′ and 3′ oligonucleotide primers homologous to nucleotides 161 to 187 and nucleotides 1093 to 1117 (LSM 12 and LSM 6, respectively) were used to amplify the variable a-helical region to construct probe LSM*pspA*12/6 (21). PCR generated DNA was purified by Gene Clean (Bio101 Inc., Vista, CA) and random prime-labeled with digoxigenin-11-dUTP using the Genius 1 Nonradioactive DNA Labeling and Detection Kit as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN).

DNA electrophoresis:

For Southern blot analysis, approximately 10μg of chromosomal DNA was digested to completion with a single restriction endonuclease, (*Hind* III, *Kpn* 1, *EcoR* 1, *Dra* 1, or *Pst* 1) then electrophoresed on a 0.7% agarose gel for 16-18 hours at 35 volts. For PCR analysis, 5ul of product were incubated with a single restriction endonuclease, (*Bcl* 1, *Bam*H 1, *Pst* 1, *Sac* 1, *Eco*R 1 *Sma* 1, and *Kpn* 1) then electrophoresed on a 1.3% agrose gel for 2-3 hours at 90 volts. In both case, 1 Kb DNA ladder was used for molecular weight makers (BRL, Gaithersburg, MD) and gels were stained with ethidium bromide for 10 minutes and photographed with a ruler.

Southern blot hybridization

The DNA in the gel was depurinated in 0.25N HCL for 10 minutes, d natured in 0.5M NaOH and 1.5M NsCl for 30 minutes, and

neutralized in 0.5M Tric-HCl (pH 7.2), 1.5M NaCl and 1mM disodium EDTA for 30 minutes. DNA was transferred to a nylon membrane (Micron Separations INC, MA) using a POSIBLOT pressure blotter (Strategene, La Jolla, CA) for 45 minutes and fixed by UV irradiation. The membranes were prehybridized for 3 hours at 42°C in 50% formamide, 5X SSC, 5X Denhardt solution, 25mM sodium phosphate (pH 6.5), 0.5% SDS 3% (wt/vol) dextran sulfate and 500μg/ml of denatured salmon containing 45% formamide, 5X SSC, 1X Denhardt solution, 20mM sodium phosphate (pH 6.5), 0.5% SDS, 3% dextran sulfate, 250μg/ml denatured sheared salmon sperm DNA and about 20ng of heat-denatured diogoxigenin-labeled probe DNA. After hybridization, the membranes were washed twice in 0.1% SDS and 2X SSC for 3 minutes at room temperature. The membranes were washed twice to a final stringency of 0.1% SDS in 0.3X SSC at 65°C for 15 minutes. This procedure yields a stringency greater than 95 percent (9, 15, 22). The membranes were developed using the Genius 1 Nonradioactive DNA Labeling and Detection Kit as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN). To perform additional hybridization with other probes, the membranes were stripped in 0.2N NaOH/0.1%SDS at 40°C for 30 minutes and then washed twice in 2X SSC (23).

Polymerase Chain Reaction (PCR):

5′ and 3′ primers homologous with the DNA encoding the N- and C-terminal ends of PspA (LSM13 and LSM2, respectively) were used in these experiments (21). Amplifications were made

using Taq DNA polymerase, $MgCl^2$ and 10X reaction buffer obtained from Promega (Madison, WI). DNA used for PCR was prepared using the method previously described in this paper. Reactions were conducted in 50ml volumes containing 0.2mM of each dNTP, and 1ml of each primer at a working concentration of 50mM. $MgCl_2$ was used at an optimal concentration of 1.75mM with 0.25 units of Taq DNA polymerase. Ten to thirty ng of genomic DNA was added to each reaction tube. The amplification reactions were performed in a thermal cycler (M.J. Research, Inc.) using the following three step program. Step 1 consisted of a denaturing temperature of 94°C for 2 minutes. Step 2 consisted of 9 complete cycles of a denaturing temperature of 94°C for 1 minute, an annealing temperature of 50°C for 2 minutes, and an extension temperature of 72°C for 3 minutes. Step 3 cycled for 19 times with a denaturing temperature of 94°C for 1 minute, an annealing temperature of 60°C for 2 minutes, and an extension temperature of 72°C for 3 minutes. At the end of the last cycle, the samples were held at 72°C for 5 minutes to ensure complete extension.

Band size estimation:

Fragment sizes in the molecular weight standard and in the Southern blot hybridization patterns were calculated from migration distances. The standard molecular sizes were fitted to a logarithmic regression model using Cricket Graph (Cricket Software, Malvern, PA). The molecular weights of the detected bands were estimated by entering the logarithmic line equation

obtained by Cricket Graph into Microsoft Excel (Microsoft Corporation, Redmond, WA) in order to calculate molecular weights based in migration distances observed in the Southern blot.

Table 15.

Restriction Enzyme	Strains Examined					Restriction Fragments (sizes in kilobases)	
	MC25	MC26	MC27	MC28	RX1	MC25-MC28	RX1
Hind III	+	+	+	+	+	7.7, 3.6	9.1, 4.2
Kpn I	+	+	+	+	+	11.6, 10.6	10.6, 9.8
*Eco*R I	+				+	8.4, 7.6	7.8, 6.6
Dra I	+				+	2.1, 1.1	1.9, 0.9
Pst I	+				+	>14, 6.1	10.0, 4.0

Table 16. Penicillin Resistant Capsular Serogroup 6 Strains from Spain

Isolate	Penicillin MIC (μg/ml)	Year	Site	Hospital
MC25	1	1986	sputum	Bellvitge
MC26	4	1988	ear	San Juan de Dios
MC27	1	1988	ear	San Juan de Dios
MC28	2	1988	?	San Juan de Dios

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21. **Use of PCR to amplify pspAs and fragments of pspAs: Example 5, infra.**

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EXAMPLE 5 - Use of PCR To Amplify *pspA*s and Fragm nts of *pspA*

In this example, Applicants used oligonucleotides derived from the DNA sequence of *pspA* of *S. pneumoniae* Rx1 both as hybridization probes and as primers in the polymerase chain reaction to investigate the genetic variation and conservation of the different regions of *pspA* and *pspA*-like sequences. The probes used ranged in size from 17 to 33 bases and included sequences representing the minus 35, the leader, the α-helical region, the proline-rich regions, the repeat regions, and the C-terminus. Applicants examined 18 different isolates representing 12 capsular and 9 PspA serotypes. The proline-rich, repeat, and leader, regions were highly conserved among *pspA* and *pspA*-like sequence.

In the previous Example, it was shown that strain Rx1 and most other strains of *S. pneumoniae* had two homologous sequences that could hybridize with probes encoding the N terminal and C terminal halves of PspA. This conclusion that these were separate sequences was supported by the fact that no matter which restriction enzymes was used there were always at least two (generally two sometimes three or four) restriction fragments of Rx1 and most other strains hybridized with the *pspA* probes. When the genome of Rx1 was digested with *Hind*III and hybridized with these, two *pspA*-homologous sequences were found to be in 4.0 and 9.1 kb fragments. Using derivative of Rx1 which had insertion mutations in *pspA*, it was possible to determine

that the 4.0 kb fragment contained the functional *pspA* sequence. The *pspA*-homologous sequence included within the 9.1 kb band was referred to as the *pspA*-like sequence. Whether or not the *pspA*-like sequences makes a product is not know, and none has been identified *in vitro*. Since *pspA*-specific mutants can be difficult to produce in most strains, and exist for only a limited number of pneumococcal isolates, this Example identifies oligonucleotide probes that could distinguish between the *pspA* and *pspA*-like sequences.

The purpose of this Example was to further define both the conserved and variable regions of *pspA*, and to determine whether the central proline-rich region is variable or conserved, and identify those domains of *pspA* that are most highly conserved in the *pspA*-like sequence (and ergo, provide oligonucleotides that can distinguish between the two). Oligonucleotides were used and are therefore useful as both hybridization probes and as primers for polymerase chain reaction (PCR) analysis.

Hybridization with oligonucleotide probes.

The oligonucleotides used in this study were based on the previously determined sequence [Yother, 1992] of Rx1 PspA. Their position and orientation relative to the structural domains of Rx1 PspA are shown in Figure 7. The reactivity of these oligonucleotide probes with the *pspA* and *pspA*-like sequences was examined by hybridization with a *Hind*III digest of Rx1 genomic DNA (Table 17). As expected, each of the eight probes recognized

the *pspA*-containing 4.0 kb fragment of the *Hind*III digested Rx1 DNA. Five of the 8 probes (LSM1, 2, 3, 7, and 12) could also recognize the *pspA*-like sequence of the 9.1 kb band at least at low stringency. At high stringency four of the probes (LSM2, 3, 4 and 5) were specific for the 4.0 kb.

These 8 probes were used to screen *Hind*III digest of the DAN from 18 strains of *S. pneumoniae* at low and high stringency. For comparison to earlier studies each of the strains was also screened using a full-length *pspA* probe. Table 23 illustrates the results obtained with each strain at high stringency. Table 18 summarizes the reactivities of the probes with the strains at high and low stringency. Strain Rx1 is a laboratory derivative of the clinical isolate, D39. The results obtained with both strains were identical. They are listed under a single heading in Table 23 and are counted as a single strain in Table 28. Although AC17 and AC94 are related clinical isolates (Swiatlo et al. unpublished), they have distinguishable *pspA*s and are listed separately. All of the other strains represent independent isolates.

The only strain not giving at least two *pspA*-homologous *Hind*III fragments was WU2. This observation was expected since WU2 was previously shown to have only one *pspA*-homologous sequence and to give only a single *Hind*III fragment that hybridizes with Rx! *pspA*. Even at high stringency 6 of the 8 probes detected more than one fragment in at least one of the 18

strains Tabl s 18 and 23. Probes LSM7, 10 and 12 reacted with DNA from a majority of the strains and detected two fragments in over 59% of the strains they reacted with. In almost every case the fragments detected by the oligonucleotide probes were identical in size to those detected by the full-length *pspA* probe. Moreover, the same pairs of fragments were frequently detected by probes from the 3′ as well as the 5′ half Rx1 *pspA*. These results are consistent with earlier findings [McDaniel, 1992] that the pairs of *Hind*III fragments from individual isolated generally include two separate but homologous sequences, rather than fragments of a single *pspA* gene.

The differences in the frequency with which the oligonucleotides reacted with (at least one fragment) of the strains in the panel was significant at $P < 0.0001$ by 2 x 8 chi square). When the oligonucleotides were compared in terms of their ability to react with both fragments of each strain the P value was also < 0.0001. Table 18 gives the percentage of strains reactive with each probe, the percentage in which only one fragment was reactive, and the percentage in which two (or more) fragments were reactive.

The last column in Table 18 give the ratio of strains that showed one reactive *Hind*III fragment at high stringency divided by the total number of reactive strains, In this column values of 1 were obtained with probes that only reacted with one band in each reactive strain. Such probes are assumed to be

those that are most specific for *pspA*. The lowest values were obtained with probes that generally see two bands in each strain. Such probes are assumed to be those that represent regions relatively conserved between the *pspA* and *pspA*-like sequences. At high stringency, probes LSM3 and LSM4 detected only a single *Hind*III fragment in the DAN of strains they reacted with. These findings suggested probes LSM3 and LSM4 were generally detecting alleles of *pspA* rather than the *pspA*-like sequence. The observation that the fragments detected by LSM3 or LSM4 were also detected by all of the other reactive probes, strengthened the conclusion that these probes generally detected the *pspA* rather than the *pspA*-like sequence. WU2 has only one *pspA*-homologous DNA sequence and secretes a serologically detectable PspA. The fact that LSM3 reacts with the single *Hind*III fragment of WU2 is consistent with the interpretation that LSM3 detects the *pspA* sequences. Sequences representing the second proline region (LSM1) and the C-terminus (LSM2) appeared to also be relatively specific for the *pspA* sequences since they were generally detected in only one of the *Hind*III fragments of each strain.

Oligonucleotides, LSM12, and LSM10 detected the most conserved epitopes of *pspA* and generally reacted with both *pspA*-homologous fragments of each strain (Table 18). LSM7 was not quite as broadly cross-reactive but detected two PspAs in 41% of strains including almost 60% of the strains it reacted with. Thus, sequences representing the leader, first proline region,

and th repeat region appear to be relatively conserved not only within *pspA* but between the *pspA* and *pspA*-like sequences. LSM3, 4, and 5 reacted with the DNA from the smallest fraction of strains of any oligonucleotide (29 - 35 percent), suggesting that the portion of *pspA* encoding the α-helical region is the least conserved region of *pspA*.

With two strains BG85C and L81905, the oligonucleotides detected more than two *Hind*III fragments containing *pspA*-homologous sequences. Because of the small size of the oligonucleotide probes and the absence of *Hind*III restriction sites within any of them, it is very unlikely that these multiple fragments were the results of fragmentation of the target DNA within the probed regions. In almost every case the extra oligonucleotides were detected at high stringency by more than one oligonucleotide. These data strongly suggest that at least in these two strains there are 3 or 4 sequences homologous to at least portions of the *pspA*. The probes most reactive with these additional sequences are those for the leader, the α-helical region and the proline rich region. The evidence for the existence of these additional *pspA*-related sequences was strengthened by results with BG58C and L81905 at low stringency where the LSM3 (α-helical) primer picked up the extra 1.2 kb band of L81905 (in addition to the 3.6 kb band) and the LSM7 (proline-rich) primer picked up the extra 3.2 and 1.4 kb bands (in addition to the 3.6 kb band) of BG58C.

Amplification of *pspA*

The utility of these oligonucleotides as PCR primers was examined by determining if they could amplify fragments of *pspA* from the genomic DNA of different pneumococcal isolates. Applicants attempted to amplify *pspA*s from 14 diverse strains of *S. pneumoniae* comprising 12 different capsular types using primers based on the Rx1 *pspA* sequence. Applicants observed that the 3′ primer LSM2, which is located at the 3′ end of *pspA*, would amplify an apparent *pspA* sequence from each of the 14 pneumococcal strains when used in combination with LSM1 located in the region of *pspA* encoding the proline-rich region (Table 19). LSM2 was also used in combination with four other 5′ primers LSM1, 3, 7, 8 and 12. LSM8 is located 5′ of the *pspA* start site (near the -35 region).

If a predominant sequence of the expected length was amplified that could be detected on a Southern blot with a full-length *pspA* probe, we assumed that *pspA* gene of the amplified DNA had homologous sequences similar to those of the *pspA* primers used. Based on these criteria the primer representing the α-helical sequence was found to be less conserved than the primers representing the leader, proline, and C-terminal sequences. These results were consistent with those observed for hybridization. The lowest frequency of amplification was observed with LSM8 which is from the Rx1 sequence 5′ of the *pspA*

start site. This oligonucleotide was not used in the hybridization studies.

Further evidence for variability comes from differences in the sizes of the amplified *pspA* gene. The Example showed that when PCR primers LSM12 and LSM2 were used to amplify the entire coding region of PspA, PCR products from different pneumococcal isolates ranged in size from 1.9 and 2.3 kb (Table 20). The regions within *pspA* encoding the α-helical, proline-rich, and repeats were also amplified from the same isolates. As seen in Table 20, the variation in size of *pspA* appeared to come largely from variation in the size of *pspA* encoding encodes the α-helical region.

Using probes that consisted of approximately the 5′ and 3′ halves of *pspA* it has been determined that the portion of *pspA* that encodes the α-helical regions is less conserved than the portion of *pspA* that encodes the C-terminal half of the molecule. This Example show using 4 oligonucleotide probes from within each half of the DNA encoding PspA. Since a larger number of smaller probes were used, Applicants have been able to obtain a higher resolution picture of conserved and variable sequences within *pspA* and have also been able to identify regions of likely differences and similarities between *pspA* and the *pspA*-like sequences.

The only strains in which the *pspA* gene has been identified by molecular mutations are Rx1, D39 and WU2 [McDaniel,

1992; Yother, 1992]. Rx1 and D39 apparently have identical *pspA* molecules [Crain, 1990; McDaniel, 1992] that are the result of the common laboratory origin of these two strains. WU2 lacks the *pspA*-like gene. Thus, when most pneumococci are examined by Southern blotting using full length-*pspA* as a probe, it is not possible to distinguish between the *pspA* and *pspA*-like loco, since both are readily detected. A major aim of these studies was to attempt to identify conserved and variable regions within the *pspA* and *pspA*-like loci. A related aim was to determine whether probes based on the Rx1 *pspA* could be identified that would permit one to differentiate *pspA* from the *pspA*-like sequence. Ideally such probes would be based on relatively conserved portion of the *pspA* sequence that was quite different in the *pspA*-like sequence. A useful *pspA* specific probe would be expected to identify the known Rx1 and WU2 *pspA* genes and identify only a single *Hind*III fragment in most other strains. Two probes (LSM3 and LSM4) never reacted with more than one *pspA*-homologous sequence in any particular strain. Both of reacted with Rx1 *pspA* and LSM3 reacted with WU2 *pspA*. Each of these probes reacted with 4 of the other 15 strains. When these probes identified a band, however, the band was generally also detected by all other Rx1 probes reactive with that strain's DNA. Additional evidence that the LSM3 and LSM4 were restricted to reactivity with *pspA* was that they reacted with the same bands in all three non-Rx1 strains. Each probe identifies *pspA* in certain

strains and even when used in combination they recognized *pspA* in over 40 percent of strains. Probes for the second proline-rich region (LSM1) and the C-terminus of *pspA* (LSM2) generally, but not always, identified only one *pspA*-homologous sequence at high stringency. Collectively LSM1, 2, 3, and 4 reacted with 16 of the 17 isolates and in each case revealed a consensus band recognized by most to all of the reactive probes.

By making the assumption that in different strains the Rx1 *pspA* probes are more likely to recognize *pspA* than the *pspA*-like sequences, it is possible to make some predictions about areas of conservation and variability within the *pspA* and *pspA*-like sequences. When a probe detected only a single *pspA*-homologous sequence in an isolate, it was assumed that it was *pspA*. If the probe detected two *pspA*-homologous sequences, it was assumed that it was reacting with both the *pspA* and *pspA*-like sequence. Thus, the approximate frequency with which a probe detects *pspA* can be read from Table 18 as the percent of strains where it detects at least one *pspA*-homologous band. The approximate frequency with which the probes detect the *pspA*-like sequence is the percent of strains in which two or more *pspA*-homologous band are detected.

Using these assumptions the most variable portion of portion of the *pspA* gene was observed to be the -35 region and the portion ncoding α-helical region. The most conserved portion of *pspA* was found to be the repeat region, the leader and

the proline rich region. Although only one probe from the region was used, the high degree of conservation among the 10 repeats in the Rx1 sequence [Yother, 1992] makes it likely that other probes for the repeat regions give similar results.

The portion of the *pspA*-like sequence most similar to Rx1 *pspA* was that encoding the leader sequence, the 5′ portion of the proline rich region, and the repeat region, and those portions encoding the N-terminal end of the proline-rich and repeat regions. The repeat region of PspA has been shown to be involved in the attachment to PspA to the pneumococcal surface. The conservation of the repeat region among both *pspA* and *pspA*-like genes suggests that if is PspA-like protein is produced, that it may have a surface attachment mechanism similar to that of PspA. The need for a functional attachment site may explain the conservation of the repeat region. Moreover, the conservation in DNA encoding the repeat regions of the *pspA* and *pspA*-like genes suggests that the repeat regions may serve as a potential anti-pneumococcal drug target. The conservation in the leader sequence between *pspA* and the *pspA*-like sequence was also not surprising since similar conservation has been reported for the leader sequence of other gram positive proteins, such as M protein of group A streptococci [Haanes-Fritz, 1988]. It is noteworthily, however, that there is little evidence at the DNA level that the PspA lead is shared by many genes other than PspA and the possible gene product of the *pspA*-like locus.

Although the region encoding the C-terminus of *pspA* (LSM12) or the 3′ portion of the proline-rich sequence (LSM1) appear to be highly conserved within *pspA* genes, corresponding regions in the *pspA*-like sequences are either lacking, or very distinct from those in *pspA*. The reason for conservation at these sites is not apparent. In the case of the PspA, its C-terminus does not appear to be necessary for attachment, since mutants lacking the C-terminal 49 amino acids are apparently as tightly attached to the cell surface as those with the complete sequence. Whether these difference from *pspA* portends a subtle difference in the mechanism of attachment of proteins produced by these two sequences in unknown. If the C-terminal end of the *pspA*-like sequence, or the 3′ portion of the proline-rich sequence in the *pspA*-like sequence are as conserved within the *pspA*-like family of genes as it is within *pspA*, then this region of *pspA* and the *pspA*-like sequence serve as targets for the development of probes to distinguish between all *pspA* and *pspA*-like genes.

With two strains, some of the oligonucleotide probes identified more than two *pspA*-homologous sequences. In the case of each of these strains, there was a predominant sequence recognized by almost all of the probes, and two or three additional sequences that were each recognized by at least two of the probes. One interpretation of the data is that there may be more than two *pspA*-homologous genes in some strains. The

significance of such sequences is far from established. It is of interest however, that although the additional sequences is far from established. It is of interest however, that although the additional sequences share areas of homology with the leader, α-helical, and proline region, they exhibited no homology with the repeat region of the C-terminus of *pspA*. These sequences, thus, might serve as elements that can recombine with *pspA* and/or the *pspA*-like sequences to generate sequence diversity. Alternatively the sequences might produce molecules with very different C-terminal regions, and might not be surface attached. If these *pspA*-like sequences make products, however, they, like PspA, may be valuable as a component of a pneumococcal antigenic, immunological vaccine compositions.

Bacterial strains, growth conditions and isolation of chromosomal DNA.

S. pneumoniae strains used in this study are listed in Table 5. Strains were grown in 100 ml of Todd-Hewitt broth with 0.5% yeast extract at 37°C to an approximate density of $5x10^8$ cells/ml. Following harvesting of the cells by centrifugation (2900xg, 10 minutes), the DNA was isolated as previously described [McDaniel, 1992#536] and stored at 4°C in TE (10mM Tris, 1mM EDTA, pH 8.0).

Amplification of *pspA* sequences.

Polymerase chain reaction (PCR) primers, which were also used as oligonucleotide probes in Southern hybridizations,

were designed based on the sequence of *pspA* from pneumococcal strain Rx1 [Yother, 1992]. These oligonucleotides were obtained from Oligos Etc. (Wilsonville, OR) and are listed in Table 22.

PCRs were done with a MJ Research, Inc., Programmable Thermal Cycler (Watertown, MA) as previously described using approximately 10 ng of genomic pneumococcal DAN with appropriate 5′ and 3′ primer pair. The sample was brought to a total volume of 50 μl containing a final concentration of 50mM KCl, 10mM Tris-HCl (PH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 0.5 mM each primer, 200mM of each deoxynucleotide triphosphate, and 2.5 U of *Taq* DNA polymerase. Following overlaying of the samples with 50 μl of mineral oil, the samples were denatured at 94°C for 2 minutes. Then the samples were subjected to 10 cycles consisting of 1 minute at 94°C, 2 minutes at 50°C, and 3 minutes at 72°C followed by another 20 cycles of 1 minute at 94°C, 2 minutes at 50°C, and 3 minutes at 72°C followed by another 20 cycles of 1 minute at 94°C, 2 minutes at 60°C, and 3 minutes at 72°C. After all 30 cycles, the samples were held at 72°C for an additional 5 minutes prior to cooling to 4°C. The PCR products were analyzed by agarose gel electrophoresis.

DNA hybridization analysis.

Approximately 5μg of chromosomal DNA was digested with *Hind*III as per the manufacturer's instructions (Promega, Inc., Madison, WI). The digested DNA was electrophoresesed at 35 mV

overnight in a 0.8% agarose gels and then vacuum-blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH).

Labeling of oligonucleotide with and detection of probe-target hybrids were both performed with the Genius System according to the manufacturer's instructions (Mannheim, Indianapolis, IN). All hybridizations were done for 18 hours at 42°C without formamide. By assuming that 1% base-pair mismatching results in a 1°C decrease in T*m* designations of "high" and "low" stringency were defined by salt concentration and temperature of post-hybridization washes. Homology between probe and target sequences was derived using calculated T*m* the established method. High stringency is defined as 90% or greater homology, and low stringency is 80-85% sequence homology.

Table 17. Hybridization of oligonucleotides with *Hin*dIII restriction fragments of Rx1 DNA.

Oligonucleotide	Region	Stringency	
		Low	High
LSM12	Leader	N.D.	4.0, 9.1
LSM5	α-helix	N.D.	4.0
LSM3	α-helix	4.0, 9.1	4.0
LSM4	α-helix	4.0	4.0
LSM7	Proline	4.0, 9.1	4.0, 9.1
LSM1	Proline	4.0, 9.1	4.0, 9.1
LSM10	Repeats	N.D.	4.0, 9.1
LSM2	C-terminus	4.0, 9.1	4.0

Note. Values indicated are the sizes of restriction fragments expressed as kb.

Table 18. Summary of Hybridization at High and Low Stringency of 8 Oligonucleotides with *Hin*dIII Restriction Fragments of the 17 Pneumococcal Isolates Listed in Figure ?

Oligonucleotide	Percent with ≥1 band		Percent with ≥2 bands		Percent with 1 band		1 band/ ≥1 band	
	Low	High	Low	High	Low	High	Low	High
LSM12		82		59		24		0.29
LSM5		29		18		12		0.40
LSM3	65	35	41	0	24	35	0.36	1.00
LSM4	35	29	0	0	35	29	1.00	1.00
LSM7	94	71	71	41	24	29	0.25	0.42
LSM1	100	65	53	12	47	53	0.47	0.82
LSM10		94		59		35		0.37
LSM2	88	53	41	12	47	41	0.53	0.78

Note, for all values listed all 17 strains were examined. If no value is listed, then no strains were examined.

Table 19. Amplification of Pneumococcal Isolates using the Indicated 5' Prime Combination with the 3′ Primer LSM2 at the 3′ end of *pspA*

5′ Primer	Region	Nucleotide Position	Amplified/ Tested	Percent Amplified
LSM8	- 35	47 to 70	2/14	14
LSM12	leader	162 to 188	8/14	57
LSM3	α-helical	576 to 598	3/14	21
LSM7	proline	1093 to 1117	12/14	86
LSM1	proline	1312 to 1331	14/14	100

Note, by 2x5 chi square analysis the differnet primers amplified different frequencies of pspAs ($P < 0.0001$). The tendency for there to be more amplification with the 3' most primers was significant at $P < 0.0001$.

Table 2D Size of amplified *pspA* fragments in kilobases

pspA Region	Primer Pairs	number *pspAs* examined	Size	Range	S.D.
Full length	LSM12 + LSM2	9	1.9-2.3	0.4	0.17
α-helical	LSM12 + LSM6	6	1.1-1.5	0.4	0.17
Proline	LSM7 + LSM9	3	0.23	0	0
Repeats	LSM1 + LSM2	19	0.6-0.65	0.05	0.01

Note: amplification was attempted with each set of primers on a panel of 19 different pspAs. Data is shown only for *pspAs* that could be amplified with the indicated primer pairs.

Table 3 Pneumococcal strains

Strain	Relevant characteristics
WU2	Capsular type 3, PspA type 1
D39	Capsular type 2, PspA type 25
R36A	Nonencapsulated mutant of D39, PspA type 25
Rx1	Nonencapsulated variant of R36A, PspA type 25
DBL5	Capsular type 5, PspA type 33
DBL6A	Capsular type 6A, PspA type 19
A66 1?/2	Capsular type 3, PspA type 13
AC94	Capsular type 9L, PspA type 0
AC17	Capsular type 9L, PspA type 0
AC40	Capsular type 9L, PspA type 0
AC107	Capsular type 9V, PspA type 0
AC100	Capsular type 9V, PspA type 0
AC140	Capsular type 9N, PspA type 18
D109-1B	Capsular type 23, PspA type 12
BG9709	Capsular type 9, PspA type 0
BG58C	Capsular type 6A, PspA type ND
L81905	Capsular type 4, PspA type 25
L82233	Capsular type 14, PspA type 0
L82006	Capsular type 1, PspA type 0

1 2

Table 2 PCR primers.

Primer	Sequence (5′ to 3′)
LSM1	CCGGATCCAGCTCCTGCACCAAAAAC
LSM2	GCGCGTCGACGGCTTAAACCCATTCACCATTGG
LSM3	CCGGATCCTGAGCCAGAGCAGTTGGCTG
LSM4	CCGGATCCGCTCAAAGAGATTGATGAGTCTG
LSM5	GCGGATCCCGTAGCCAGTCAGTCTAAAGCTG
LSM6	CTGAGTCGACTGGAGTTTCTGGAGCTGGAGC
LSM7	CCGGATCCAGCTCCAGCTCCAGAAACTCCAG
LSM8	GCGGATCCTTGACCAATATTTACGGAGGAGGC
LSM9	GTTTTTGGTGCAGGAGCTGG
LSM10	GCTATGGCTACAGGTTG
LSM11	CCACCTGTAGCCATAGC
LSM12	CCGGATCCAGCGTGCCTATCTTAGGGGCTGGTT
LSM13	GCAAGCTTATGATATAGAAATTTGTAAC

84F

Fig 2

TABLE 23

Hybridization at high stringencyh of eight different PspA probes with *Hin*dIII digests of 18 strains of *Streptococcus pneumoniae*																
Probe	**Strain**															
	Rx1/D39	WU2	DBL5	DBL6A	A66	AC94	AC17	AC40	AC107	AC100	AC140	DC109	BG9709	BG58C	L81905	L82233	L82006
FL-Rx1	4.0,9.1	3.8	3.7,5.8	3.0,3.4	3.6,4.3	3.6,6.3	3.6,6.3	3.2,3.6	3.6,6.3	4.0,8.0	3.0,4.0	3.3,4.7	2.2,9.6	1.4,3.2 3.6	3.6,5.2	3.7 8.2	4.3,6.4
LSM 12	4.0,9.1	3.8	3.7,5.8	3.0,3.4	4.3		3.6,6.3	3.2,3.6		4.0,8.0	4.0	3.3,4.7	2.2,9.6	1.4,3.2 3.6	3.6	1.3,3.7	
LSM5	4.0					3.6, 6.3							2.2,9.6	3.6	1.2,2.3 3.6		
LSM3	4.0	3.8				6.3							2.2	3.6	3.6		
LSM4	4.0												2.2	3.6	3.6	3.7	
LSM7	4.0, 9.1	3.8	3.7	3.0,3.4	3.6			3.2,3.6			3.0,4.0	3.3,4.7	2.2,9.6	3.6	2.3 3.6	3.7	
LSM1	4.0, 9.1	3.8	3.7,5.8	3.4		6.3		3.2	3.6	4.0	4.0		2.2		5.2		
LSM 10	4.0, 9.1	3.8	3.7	3.4	3.6, 4.3		3.6,6.3	3.2	3.6,6.3	4.0	4.0	3.3, 4.7	2.2, 9.6	3.2 3.6	3.6,5.2	1.3,3.7	4.3,6.4
LSM2	4.0		3.7			3.6	3.6		3.6,6.3	4.0	3.0,4.0	4.7					4.3

Note: All probes were tested versus *Hin*dIII digests of all strains. If no bands are listed none were detected. Strains Rx1 and D39 gave identical results and are shown in a singel column. The full name of strain AC109 is AC109-1B

EXAMPLE 6 - Restriction Fragm nt Length Polymorphisms of *pspA* Reveals Grouping

Pneumococcal surface A (PspA) is a protection eliciting protein of *Streptococcus pneumoniae*. The deduced amino acid sequence of PspA predicts three distinct domains; an α helical coiled-coil region, followed by two adjacent proline-rich regions, and ten 20 amino acid repeats. Almost all PspA molecules are cross-reactive with each other in variable degrees. However, using a panel of monoclonal antibodies specific for individual epitopes, this protein has been shown to exhibit considerable variability even within strains of the same capsular type. Oligonucleotide primers based on the sequence of *pspA* from *S. pneumoniae* Rx1 were used to amplify the full-length *pspA* gene and the 5′ portion of the gene including the α-helical and the proline-rich region. PCR-amplified product were digested with *Hha* I or *Sau*3A I to visualize restriction fragment length polymorphism of *pspA*. Although strains were collected from around the world and represented 21 different capsular types, isolates could be grouped into 17 families or subfamilies based on their RFLP pattern. The validity of this approach was confirmed by demonstrating that *pspA* of individual strains which are known to be clonally related were always found within a single *pspA* family.

Numerous techniques have been employed in epidemiological surveillance of pneumococci which include

serotyping, ribotyping, pulsed field electrophoresis, multilocus enzyme electrophoresis, penicillin-binding protein patterns, and DNA fingerprinting [Lefever, 1993] [Viering, 1989] [Markiewic, 1989]. Previous studies have also utilized the variability of pneumococcal surface protein A (PspA) to differentiate pneumococci [Crain, 1990]. This protein, which can elicit protective antipneumococcal antibodies, is a virulence factor found on all pneumococcal isolates [McDaniel, 1991]. Although PspA molecules are commonly cross-reactive, they are seldom antigenically identical [Crain, 1990]. This surface protein is the most serologically diverse protein know on pneumococci; therefore, it is an excellent market to be used to follow individual strains. Variations in PspA and the DNA surrounding its structural gene have proven useful for differentiation of *S. pneumoniae* [McDaniel, 1992].

When polyclonal sera are used to identify PspA, cross-reaction is observed between virtually all isolates [Crain, 1990]. Conversely, when panels of monoclonal antibodies are used to compare PspA of independent isolation they are almost always observed to express different combinations of PspA epitopes. A typing system based on this approach has limitations because it does not easily account for differences in monoclonal binding strength to different PspA molecules. Moreover, some strains are weakly reactive with individual monoclonal antibodies and may not always give consistent results.

A less ambiguous typing system that takes advantage of the diversity of PspA was therefore necessary to develop and was used to examine the clonality of strains. This method involves examination of the DNA within and adjacent to the pspA locus. Southern hybridizations of pneumococcal chromosomal DNA digested with various endonucleases, such as *Hind* III, *Dra* I, or *Kpn* I, and probed with labeled *pspA* provided a means to study the variability of the chromosome surrounding *pspA*. When genomic DNA is probed, the *pspA* and the *pspA*-like loci are revealed. In most digests the *pspA* probe hybridizes to 2-3 fragments and, digests of independent isolates were generally dissimilar.

Like the monoclonal typing system, the Southern hybridization procedure permitted the detection of clones of pneumococci. However, it did not provide a molecular approach for following *pspA* diversity. Many of the restriction sites defining the restriction fragment length polymorphism (RFLP) were outside of the *pspA* gene, and it was difficult to differentiate the *pspA* gene from the *pspA*-like locus. In an effort to develop a system to follow *pspA* diversity Applicants examined the RFLP of PCR-amplified *pspA*. Amplified *pspA* was digested with *Sau*3A I and *Hha* I, restriction enzymes with four base recognition sites. To evaluate the utility of this approach *pspA* from clinical and laboratory strains known to be clonally related as well as random isolates were examined.

Bacterial strains. Derivatives of the *S pneumoniae* D39-Rx1 family were kindly provided by Rob Massure and Sanford Lacks (Figure 8). Eight clinical isolates from Spain and four isolates from Hungary, a gift from Alexander Tomasz. Seventy-five random clinical isolates from Alabams, Sweden, Alaska, and Canada were also studied (Crain 1990, Tomaz and Crain, Waltamn).

PCR amplifications. The oligonucleotide primers used in this study are listed in Table 24. Chromosomal DNA, which was isolated according to procedures described by Dillard et al., was used as template for the PCR reactions. Amplification was accomplished in a 50 μl reaction containing approximately 50 ng template DNA, .25U Taq, 50 μM of each primer, 175 μM $MgCl_2$, and 200 μM dNTP in a reaction buffer containing 10 μM Tris-HCl, pH 9.0, 50μM KCl, 0.1% Triton X-100, 0.01% wt/vol. gelatin. The mixture was overlaid with mineral oil, and placed in a DNA thermal cycler. The amplification program consisted on an initial denaturation step at 94°C, followed by 29 cycles opf 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The final cycle included an incubation at 72°C for 5 min.

Restriction fragment analysis of PCR-amplified product. Aliquots of the PCR mixtures were digested with *Hha* I or *Sau*3A I in a final volume of 20μl according to manufacturer's protocols. After digestion the DNA fragments were electrophoresed on a 1.3% TBE agaros gel and stained with ethidium bromide. Fragment

sizes were estimated by comparison to a 1kb DNA ladder (Gibco BRL).

Because of the variability of *pspA*, and the fact that the entire *pspA* sequence is known for only one gene, it has not been possible to design primers which amplify *pspA* from 100% of pneumococcal strains. However, oligonucleotide primers, LSM2 and LSM1, can amplify an 800 bp region of the C-terminal end in 72 of the 72 stains tested. Based on hybridizations at different stringencies, this region was found to be relatively conserved in pneumococcal strains, and thus would not be expected to be optimal for following restriction polymorphisms within the *pspA* molecule. LSM13 and LSM2, primers which amplify the full length *pspA* gene, can amplify *pspA* from approximately **79%** 55/75 of the strains tested (Table 25).

Stability of amplified RFLP pattern within clonally related pneumococci

To determine the stability of *pspA* during long passages in vitro, we examined the RFLP pattern of the *pspA* gene of the derivatives of the *S. pneumoniae* D39-Rx1 family. Rx1 is an acapsular derivative of *S. pneumoniae* D39, the prototypical pneumococcal laboratory strain isolated by Avery in 1914. Throughout the 1900's spontaneous and chemical mutations have been introduced into D39 by different laboratories (Figure 8) [Smith, 1979]. During this period unencapsulated strains were maintained in vitro, and D39 was passed both in vivo and in vitro

passage. All the derivatives of D39, including Rx1, R6, RNC, and R36A, produced a 1.9 kb fragment upon PCR amplification of full length *pspA*. All members of the family exhibited the RFLP pattern. Digestion with *Sau3A* I of PCR amplified full length *pspA* revealed a .83, .58, .36 and a .27 kb fragment in all of the D39-rX1 derivatives of the family. Digesting the full length *pspA* with *Hha* I resulted in bands which were .76. .47, .39, .35, and .12 kb (Figure 9 or Table 26).

The stability of *pspA* polymorphism was also investigated using pneumococcal isolates which had previously been shown to be clonally related by other criteria, including capsule type, antibiotic resistance, enzyme electromorph, and PspA serotype. Three sets of isolates, all of which were highly penicillin resistant, were collected from patients during an outbreak in Hungary and two separate outbreaks in Spain. PCR amplified full length *pspA* from the capsular type 19A pneumococcal strains from the outbreak in Hungary, DB18, DB19, DB20, and DB21, resulted in a band approximately 2.0 kb. After digesting full length *pspA* with *Hha* I, four fragments were visualized., 89, .48, and .28 kb. Digestion with *Sau*3A I yielded five fragments .880, .75, .35, .34, and .10kb. Capsule type 6B pneumococcal strains, DB1, DB2, DB3, and DB4, were obtained from an outbreak in Spain. Full length *pspA* from these strains were approximately 1.9 kb. Digestion of the PCR-amplified fragment with *Hhs* I resulted in four fragments which were .83, .43, .33,

and .28 kb. *Sau*3A I digestion yield a .88, .75, .34, and .10 kg fragments. DB6, DB8, and DB9, which are capsular serotype 23F strains, were isolated from a second outbreak in Spain. DB6, DB8, and DB9 had an amplified *pspA* product which was 2.0 kb. *Hha* I digested fragments were .90, .52, .34, and .30 kb and *Sau*3A I fragments were .75, .52, .39, .22, .20, and .10 kb in size (Figure 10). DB7 had a 19A capsular serotype and was not identical to DB6, DB8, and DB9. In the D39/Rx1 family and in each of the three outbreak families the size of the fragments obtained from the *Hha* I and the *Sau*3A I digests totaled approximately 2.0 kb which is expected if the amplified product represents a single *pspA* sequence.

Diversity of RFLP pattern of amplified pspA from random pneumococcal isolates

PCR amplification of the *pspA* gene from 70 random clinical pneumococcal isolates yielded full-length *pspA* ranging in size from 1.8 kb to 2.3 kb. RFLP analysis of PCR-derived *pspA* revealed two to six DNA fragments ranging in size from 100 bp to 1.9 kb depending on the strain. The calculated sum of the fragments never exceeded the size of the original amplified fragment. Not all pneumococcal strains had a unique *pspA*, and some seemingly unrelated isolates from different geographical regions and different capsular types exhibited similar RFLP patterns. Isolates were grouped into families based on the

number of fragments produced by *Hha* I and *Sau*3A I digests and the relative size of these fragments.

Based on the RFLP patterns it was possible to identify 17 families with four of the families containing pairs of subfamilies. Within families all of the restriction fragments were essentially the same regardless which restriction enzyme was used. The subfamilies represent situations where two families share most but not all the restriction fragments. With certain strains an FRLP pattern was observed where detectable fragment size differed from the pattern of the established family by less than 100 bp. Since the differences were considered small compared to the differences in the fragment size and the number of fragments between families, they were not considered in family designation. The RFLP pattern of two isolates from six of the families is pictured in Figure 11, Table 27. These families were completely independent of the capsular type or the protein type as identified by monoclonal antibodies (Table 28 and 29).

Previous DNA hybridization studies have demonstrated that the *pspA* gene of different isolates are the most conserved in their 3′ region of the gene and more variable in the 5′ region of the gene. Thus, if seemed likely that the differences in the *pspA* families reflected primarily differences in the 5′ end of the gene. To confirm this theory, the α helical and proline region of *pspA* was examined without the amino acid repeats. Nucleotide primers LSM13 and KSH2 were used to amplify this

fragment which is approximately 1.6 kb. Examination of this region of *pspA* afforded two things.

This primer pair permitted amplification of 90% of the strains which is greater than the 75% of the strains which can be amplified with oligonucleotides which amplify the full length gene. Second, it allowed Applicants to examine if the original groupings which were based on the full length gene coincide with the fingerprint patterns obtained by looking at the 5′ half of the gene.

Figure 12 contains the same strains which were examined in Figure 11 but the PCR products were amplified with SKH2 and LSM13. The RFLP patterns obtained from digestion of the amplified α helical and proline rich region confirms the original designated families. However, these primers amplify a smaller portion of the *psaA* and therefore the difference is the families is not as dramatic as the RFLP patterns obtained from the RFLP pattern of the full length gene.

The polymerase chain reaction has simplified the process of analyzing *pspA* gene and have provided a means of using *pspA* diversity to examine the epidemiology of *S. pneumoniae*. Because not all strains contained a unique fingerprint of *pspA*, RFLP patterns of *pspA* cannot be used alone to identify the clonality of a strain. Our results indicate the RFLP of PCR-amplified *pspA* from pneumococcal strains in conjunction with other techniques may be useful for identifying the clonal

relatedness among pneumococcal isolates, and that this pattern is stable over long passages in vitro.

These findings suggests that the population of *pspA* is not as diverse as originally believed. PCR-RFLP of *pspA* may perhaps represent a relatively simplistic technique to quickly access the variability of the gene within a population. Further, these findings enable techniques to diagnose. *S. pneumoniae* via PCR or hybridization by primers on probes to regions of *pspA* common within groupings.

The sequence studies divide the known strains into several families based on sequence homologies. Sequence data demonstrates that there have been extensive recombinations occurring in nature within *pspA* genes. The net effect of the recombination is that the "families" identified by specific sequences differ dependin upon which part of the *pspA* molecule is used for analysis. "Families" or "grouping identified by the 5' half of the alpha-helical region, the 3' half of the α-helical regio and the proline rich region are each distinct and differ slightly from each other. In addition there is considerable evidence of other diversity (including base substitutic and deletions and insertions in the sequences) among otherwise closely related molecules.

This result indicates that it is expected that there will be a continuum of overlappin sequences of PspAs, rather than a discrete set of sequences.

The findings indicate that there is the greatest conservation of sequence in the 3' ha of the α-helical region and in the immediate 5' tip. Because the diversity in the mid half of the a-helical region is greater, this region is of little use in predicting cross-reactivity among vaccine components and challenge strains. **Thus, the sequence of 3' half of the alpha-helical region and the 5' tip of the coding sequence are likely to the critical sequences for predicting PspA cross-reactions and vaccine composition.**

The sequence of the proline-rich region may not be particularly important to composition of a vaccine because this region has not been shown to be able to elicit cross-protection even though it is highly conserved. The reason for this is presumably because antibodies to epitopes in this region are not surface exposed.

Based on our present sequences of 27 diverse *pspAs* we have found that there are 4 families of the 3' half of the α-helical region and 2-3 families of the very 5' tip the α-helical region. Together these form 6 combinations of the 3' and 5' families. This approach therefore should permit us to identify a panel of *pspAs* with 3' and 5 helical sequences representative of the greatest number of different *pspAs*. See Fig 13.

Table 3½. Oligonucleotides used in this study.

Designation	Sequence 5'-3'	Nucleotide position
LSM2	GCG CGT CGA CGG CTT AAA CCC ATT CAC CAT TGG	1990 to 1967
LSM1	CCG GAT CCA GCT CCT GCA CCA AAA AC	1312 to 1331
LSM13	GCA AGC TTA TGA TAT AgA AAT TTG TAA C	1 to 26
SKH2	CCA CAT ACC GTT TTC TTG TTT CCA GCC	1333 to 1355

95A

Table 25. Amplification of *pspA* from a panel of 72 independent isolates* of *S. pneumoniae*.

CAPSULE TYPE	NUMBER OF STRAINS EXAMINED	LSM13 AND LSM2	LSM13 AND SKH2
		% OF STRAINS AMPLIFIED	% OF STRAINS AMPLIFIED
1	3	100	100
2	1	100	100
3	8	50	87
4	6	67	100
5	1	100	100
6	7	29	86
6A	2	100	100
6B	6	100	100
7	2	50	100
8	1	100	100
9V	3	100	100
9A	2	100	100
9L	1	100	100
9N	3	100	100
10	1	100	100
11	2	50	100
12	2	0	100
13	1	100	100
14	4	0	75
15	2	50	50
19	5	100	100
22	3	33	100
23	1	100	100
33	1	0	100
35	1	0	100
nd	3	100	100

*Our strain collection contains several groups of isolates known to be previously to be clonal and collected for that purpose. The data reported in the table includes only one representative isolate from such clonal groups.

Table 36. Rx1-D39 derivatives

ISOLATE	SIZE OF *Hha* I DIGESTS (Kb)	SIZE OF *Sau*3A I DIGESTS (Kb)
D39	.76, .47, .39, .35, .12	.83, .58, .36, .27
Rx1	.76, .47, .39, .35, .12	.83, .58, .36, .27
R800	76, .47, .39, .35, .12	.83, .58, .36, .27
R6	76, .47, .39, .35, .12	.83, .58, .36, .27
R61	76, .47, .39, .35, .12	.83, .58, .36, .27
R6X	.76, .47, .39, .35, .12	.83, .58, .36, .27
R36NC	.76, .47, .39, .35, .12	.83, .58, .36, .27
R36A	.76, .47, .39, .35, .12	.83, .58, .36, .27

TABLE 27. Strain information and family designation of independ nt isolates.

STRAIN	CAPSULE TYPE	PspA TYPE	FAMILY	SIZE OF *Hha* I FRAGMENTS	SIZE OF *Sau*3A I FRAGMENTS
BG9163	6B	21	C	1.55, .35	1.05, .35, .22
EF6796	6A	1	C	1.5, .35	1.05, .35, .22
EF5668	4	12	DD	1.25, .49, .32	1.0, .80, .35
EF8616A	4	ND	DD	1.25, .49, .32	1.0, .80, .35
EF3296	4	20	E	1.0, .40, .33	1.15, .50, .34
EF4135	4	ND	E	1.0, .40, .33	1.15, .50, .34
BG7619	10	ND	F	1.3, .40, .29, .10	.82, .76, .35
BG7941	11	ND	F	1.3, .40, .29, .10	.82, .76, .35
BG7813	14	8	H	1.05, .70, .36	.90, .77, .35
BG7736	8	ND	H	1.05, .70, .36	.90, .77, .35
AC113	9A	ND	I	1.4, .34, .28	1.2, .80
AC99	9V	5	I	1.4, .34, .28	1.2, .80

Table 8. Relationship of RFLP family and PspA type.

RELATIONSHIP BETWEEN PSPA TYPE AND RFLP FAMILY																					
	PspA Type																				
pspA FAMILY	0	1	3	5	8	12	13	16	18	19	20	21	24	25	26	30	33	34	36	37	ND
A		1					1														
B														1	1						1
C		2										1	1								4
D								1		1											
DD						2															
E			1								1					1					
F						1											1				4
FF														1							3
G								1											1		
H	1				1			1		1											5
I	3			1				2	2												1
II																	1				
J	4								1	1										1	3
K	1									1				1							
KK	1																			1	3
L								1													1
M								1	1									1			1
MM						1															

Table 29. Relationship of Capsular type and RFLP family.

RELATIONSHIP BETWEEN CAPSULAR TYPE AND RFLP FAMILY																										
	Capsule Type																									
pspA Family	1	2	3	4	5	6	6A	6B	7	8	9A	9L	9N	9V	10	11	12	13	14	15	19	22	23	31	33	35	ND
A			3																								
B			1	1																							
C						2	1	2														2					1
D			1				1																				
DD				2																							
E			1	2		1																					
F						1											1						3				1
FF			1			1									1	1											
G																1					1						
H			1			1			2	1							1		1	1	1						
I											2		2	4													
II					1																						
J	2					2						1	1					1	2		2						1
K		1																					1		1		
KK	1					1		1											1			1					
L																							1				1
M								1																1			
MM																					1						

EXAMPLE 7 - Ability of PspA immunogens to protect against individual challenge strains

Protocol: CBA/N or BALB cJ mice were given 1 injection of 0.5 - μg PspA in CFA, followed 2 weeks later by a boost in saline, and challenged between 7 and 14 (average 10) days post boost. Control mice were administered a similar immunization regimen, except that the immunization came from an isogeneic strain unable to make PspA. The PspA was either full length, isolated from pneumococci or cloned full length or BC100 PspA, as little statistical significance has been seen in immunogenicity between full length PspA and BC100. The challenge doses ranged from about 10^3 to 10^4 pneunocci in inoculum, but in all cases the challenge was at least 100 times LD_{50}.

Result: The results are shown in the following Tables 30 to 60, and the conclusions set forth therein.

From the data, it appears that an antigenic, immunological or vaccine composition can contain any two to seven, preferably three to five PspA, e.g., PspAs from R36A and BG9739,

alone, or combined with any or all of PspAs from Wu2, Ef5668, and DB15. Note that surprisingly Wus PspA provided better protection against D39 that did R36a/Rx1/D39, and that also surprisingly PspA from Wu2 protected better against BG9739 than did PspA from BG9739. Combinations containing R36A, BG9739 and WU2 PspAs were most widely protective; and therefore, a preferred composition can contain any three PspA, preferably R36A, BG9739 and WU2. The data in this Example shows that PspA from varying strains is protective, and that it is possible to formulate protective compositions using any PspA or any combination of the PspAs from the eight different PspAs employed in the tests. Similarly, one can select PspaS on the basis of the groupings in the previous Example. Note additionally that each of PspA from R36A, BG9739, EF5668 and DBL5 are, from the data, good for use in compositions.

A note about use of medians rather than averages. Applicants have chosen to express data as median (a non-parametric parameter) rather than averages because the times to

death do not follow a normal distribution. In fact there are generally two peaks. One is around day 3 or 6 when most of the mice die and the other is at > 21 for mice that live. Thus, it becomes nonsensical to average values like 21 or 22 with values like 3 or 6. One mouse that lives out of 5 has a tremendous effect on such an average but very little effect on the median. Thus, the median becomes the most robust estimator of time to death of most of the mice.

Having thus described in detail certain preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above description, as many apparent variations thereof are possible without departing from the spirit or scope thereof.

TABLE 30

Relative ability of different PspAs to Protect against each challenge strains f *S. pneumoniae*

(Summary of statistically significant protection)

				Vaccine PspA									
Challenge	Caps	PspA	*pspA*	R36A, Rx1,D39	JD908/ WU2	JS1020/ BG9739	EF3296	EF5668	L81905	JS5010.3 DBL5	JS3020 DBL6A	All immune	best protect
Strain	type	type	family	K	a	b	E	DD	b	II	D	--	--
D39	2	25	K	++	+++			+				++	+++
WU2	3	1	a	+++	+++	+++		+++	+++	+++	+++	+++	+++
A66	3	13	a	+++	+++	+++		+++	+++	+++	+±	+++	+++
EF10197	3	18	M	+++		+++						+++	+++
ATCC6303	3	7	a	+++								+++	+++
BG9739	4	26	b	+	+++	+	0+	0	+±	0	0	++	+++
EF3296	4	20	E	+±	+±	0+				0	0	0	+±
EF5668	4	12	DD	+	0	+++	0+	+++	0+	+	0+	++	+++
L81905	4	23	b	+	+	++	++	0	+	+±	+±	++	++
DBL5	5	33	II	+		+		+	+	++	0	++	++
EF6796	6A	1	C	+++								+++	+++
DBL6A	6A	19	D	+++	+±	++	+±	+++	+±	+±	+++	++	+++
BG9163	6B	21	C	+++		+++						+++	+++
BG7322	6B	24	C	+++	+++	+±	0	+++	+±	+++	+±	+++	+++

Note: Empty cells indicate that no experiment has been done. Bold means significant at $P < 0.05$, Small font bold (+) means $0.02 \leq P < 0.05$. Large font bold means $P < 0.02$. For this table statistical significance refers to delay in time to death except as indicate in the (+) footnote below. When "all immune" showed significant protection against death but individual data cells did not, the result for "all immune" is presented under best protection on the assumption that if more mice were done in each data cell one or more of them would have exhibited significant protection against death.

+++ = statistically significant protection against death; ≥ 50% protection from death
++ = statistically significant protection against death; < 50% protection from death
+± = statistically significant delay in death; ≥20 protection from death
+ = statistically significant delay in death; <20 protection from death, (or significant protection against death but not a significant delay in death)
0++ = Not statistically delay in time to death; but ≥ 50% protection from death
0+ = Not statistically delay in time to death; but >1.5 day extension in median time to death or ≥20% protection from death.
0 = No apparent extension in time to death or protection from death.

TABLE 31

Relative ability of different PspAs to Protect against each challenge strains of *S. pneumoniae*

(Expressed as Median days Alive post challenge)

				Vaccine PspA									
Challenge	Caps	PspA	*pspA*	R36A, Rx1,D39	JD908 WU2	JS1020/ BG9739	EF3296	EF5668	L81905	JS5010.3 DBL5	JS3020 DBL6A	All immune	All control
Strain	type	type	family	K	a	b	E	DD	b	II	D	--	--
D39	2	25	K	4.5	>21			4				5	2
WU2	3	1	a	>21	>21	>21		>21	>21	>21	>21	>21	2
A66	3	13	a	>21	>21	>21		>21	>21	>21	4	>21	2
EF10197	3	18	M	>21		>21						>21	2
ATCC6303	3	7	a	>21								>21	5
BG9739	4	26	b	3	>21	6	3	3	5, 13	2	2	3	2
EF3296	4	20	E	5	5	4.5				2	2	3	2
EF5668	4	12	DD	6	2	>21	13	>21	4	>21	5	8	3
L81905	4	23	b	5	5	8	6	3	5	3	3.5	5	2
DBL5	5	33	II	4		3		3	3.5	6	2	3.5	2
EF6796	6A	1	C	>21								>21	1
DBL6A	6A	19	D	>21	8.5	13	9	>21	8	12	>21	12.5	5.5
BG9163	6B	21	C	>21		>21						>21	8.5
BG7322	6B	24	C	>21	>21	14.5	6	>21	12.5	>21	11	>21	7

Note: **Bold** denotes statistically significant extension of life at $P < 0.05$. **Small font denotes $0.02 \leq P < 0.05$; large font denotes $P < 0.02$.** Median times to death indicated as 8,>21, are situations where the medium as not within a continuum of values. In those cases the numbers shown are those closest to the median. In these cases the values give are those closest to the calculated median. Fractional values such as 3.5, indicate that the median is halfway between two numbers, in this case 3 and 4. As indicated in the original data (S103B), some experiments were terminated prior to 21 days post infection. There is little reason to assume, however, that results would have been significantly effected by the early termination's since very few mice infected with the strains used in those studies, have ever been observed to die later than 10 or 15 days post challenge. For statistical purposes all mice alive at the end of experiments were assumed to have been completely protected, and for the sake of calculations all surviving mice were assigned values of >21.

TABLE 32

Ability of different PspAs to Protect Against Each Challenge strain of *S. pneumoniae*

(Expressed as increase in survival time in days)

(A denotes ≥ 50% immune mice alive)

				Vaccine PspA									
				R36A,	JD908	JS1020/	EF3296	EF5668	L81905	JS5010.3	JS3020	All	Best
Challenge	Caps	PspA	*pspA*	Rx1,D39	WU2	BG9739				DBL5	DBL6A	immune	Result
Strain	type	type	family	K	a	b	E	DD	b	II	D		
D39	2	25	K	2.5	A			2				3	A
WU2	3	1	a	A	A	A		A	A	A	A	A	A
A66	3	13	a	A	A	A		A	A	A	2	A	A
EF10197	3	18	M	A		A						A	A
ATCC6303	3	7	a	A								A	A
BG9739	4	26	b	1	A	4	1	1	3, 11	0	0	1	A
EF3296	4	20	E	3	3	2.5				0	0	1	3
EF5668	4	12	DD	3	-1	A	10	A	1	A	2	5	A
L81905	4	23	b	3	3	6	4	1	3	1	1.5	3	6
DBL5	5	33	II	2		1		1	1.5	4	0	1.5	4
EF6796	6A	1	C	A								A	A
DBL6A	6A	19	D	A	3	7.5	3.5	A	2.5	6.5	A	7	A
BG9163	6B	21	C	A		A						A	A
BG7322	6B	24	C	A	A	7.5	-1	A	5.5	A	4	A	A
				R36A	WU2	BG9739	EF3296	EF5668	L81905	DBL5	DBL6A	All	Best

Note: **Bold** denotes statistically significant extension of life at $P < 0.05$. **Small font denotes $0.02 \leq P < 0.05$; large font denotes $P < 0.02$.** Median increases in survival listed as 3, 9 or 1,A denote groups where the median does not fall within a continuum of values. In these cases the values give are those closest to the calculated median. Fractional values such as 3.5, indicate that the median is halfway between two numbers, in this case 3 and 4.

TABLE 33

Relative ability of different PspAs to Protect against each challenge strains of *S. pneumoniae*

(expressed % alive at 21 days post challenge)

				Vaccine PspA									
				R36A,	JD908	JS1020/	EF3296	EF5668	L81905	JS5010.3	JS3020	All	All
Challenge	Caps	PspA	*pspA*	Rx1,D39	WU2	BG9739				DBL5	DBL6A	immune	control
Strain	type	type	family	K	a	b	E	DD	b	II	D	--	
D39	2	25	K	38	60			30				38	3
WU2	3	1	a	100	100	100		100	100	100	100	100	1.5
A66	3	13	a	75	100	80		75	100	60	20	76	5
EF10197	3	18	M	100		80						90	0
ATCC6303	3	7	a	100								100	0
BG9739	4	26	b	11	60	13	25	0	25	0	0	12	0
EF3296	4	20	E	25	20	10				0	0	8	0
EF5668	4	12	DD	22	25	60	40	100	40	60	0	41	9
L81905	4	23	b	10	0	31	40	0	0	14	0	14	0
DBL5	5	33	II	10		14		0	0	29	0	4	0
EF6796	6A	1	C	100								100	0
DBL6A	6A	19	D	67	25	33	0	60	25	0	80	35	4
BG9163	6B	21	C	89				80				86	20
BG7322	6B	24	C	100	60	25	0	89	25	80	25	55	6

Bold, denotes statistically significant protection against death at $P < 0.05$. Bold small font, indicates significant protection against death at $0.02 \leq P < 0.05$. Bold large font, indicates significant protection against death at $P < 0.02$.

TABLE 4

Relative ability of different PspAs to Protect against each challenge strain of *S. pneumoniae*

(% protected from death at 21 days post challenge)

				Vaccine PspA									
Challenge Strain	Caps type	PspA type	*pspA* family	R36A, Rx1,D39 K	WU2 JD908 a	BG9739 JS1020 b	EF3296 E	EF5668 DD	L81905 b	DBL5 JS5010.3 II	DBL6A JS3020 D	All immune –	Best result
D39	2	25	K	**36**	**59**			28				**36**	**59**
WU2	3	1	a	**100**	**100**	**100**		**100**	**100**	**100**	**100**	**100**	**100**
A66	3	13	a	**71**	**100**	**79**		**74**	**100**	**58**	16	**75**	**100**
EF10197	3	18	M	**100**		80						**90**	**100**
ATCC6303	3	7	a	**100**								**100**	**100**
BG9739	4	26	b	11	**60**	13	25	0	25	0	0	12	**60**
EF3296	4	20	E	25	20	10				0	0	8	25
EF5668	4	12	DD	14	18	56	34	**100**	34	56	-10	**35**	**100**
L81905	4	23	b	10	0	**31**	**40**	0	0	14	0	**14**	**40**
DBL5	5	33	II	10		14		0	0	**29**	0	**4**	**29**
EF6796	6A	1	C	**100**								**100**	**100**
DBL6A	6A	19	D	**66**	22	30	-4	**58**	22	-4	**79**	**33**	**79**
BG9163	6B	21	C	**86**		**75**						**83**	**86**
BG7322	6B	24	C	**100**	**57**	22	0	**88**	22	**79**	22	**52**	**100**

Bold, denotes statistically significant protection against death at $P < 0.05$. **Bold small font**, indicates significant protection against death at $0.02 \leq P < 0.05$. **Bold large font**, indicates significant protection against death at $P < 0.02$.
% protected has been corrected for any survivors in the control mice.
% protected = 100 x (% alive in immune - % alive in control)/(100 -% alive in control). Thus, if there were any mice alive in the control animals, the calculated "% protected" is less than the observed "% alive" listed in the previous table. The only exceptions to this are if 100% of immunized mice lived. Negative numbers mean that less immunized mice lived than did control mice. Please note that none of these negative numbers are significant even though we are using a one tailed test.

TABLE 35

Recommended Immunogens to Pr tection against the indicated challenge strains f *S. pneumoniae* Based on Pr tection Score Based on median days alive and percent protected
(numbers refer to preference as a vaccine strain with respect to the indicated challenge strain,
1= best)

				Vaccine PspA							
Challenge Strain	Caps type	PspA type	*pspA* family	R36A, Rx1,D39 K	WU2 JD908 a	BG9739 JS1020 b	EF3296 E	EF5668 DD	L81905 b	DBL5 JS5010.3 II	DBL6A JS3020 D
D39	2	25	K	2	1			3			
WU2	3	1	a	1	1	1		1	1	1	1
A66	3	13	a	2	1	2		2	1	3	0
EF10197	3	18	M	1		2					
ATCC6303	3	7	a	1							
BG9739	4	26	b	3	1	2	3	3	2	0	0
EF3296	4	20	E	1	1	2				0	0
EF5668	4	12	DD	0	0	2	3	1	0	2	0
L81905	4	23	b	2	0	1	1	0	0	0	0
DBL5	5	33	II	2		3		0	3	1	0
EF6796	6A	1	C	1							
DBL6A	6A	19	D	2	0	3	0	2	0	0	1
BG9163	6B	21	C	1		1					
BG7322	6B	24	C	1	2	3		1	3	1	3
Number of #1's				7	5	3	1	3	2	3	2

Bold, denotes statistically significant protection against death at $P < 0.05$. Where more than one PspA were equally protective, the same values were given to each. Recommendations are based on days to death with % protection dividing ties, especially among those where greater than 50% of mice lived to 21 days. "0" indicates test were conducted but compared to the other PspAs this one is not recommended.

Conclusions:

Statistically significant protection against death with >50% protection; 11/14 of the strains = 79%
Statistically significant protection against death; 13 / 14 strains = 93%
Statistically significant extension of life in 14/14 or 100% of strains.

TABLE 36

Best Choice for Vaccine Components as of 95/8/27						
	Vaccine Component (cumulative strains protected) % maximally protected					
Criterion	1	2	3	4	5	6
≥ #1 PspA for each challenge strain	R36A (7) 50%	WU2 (10) 71%	BG9739* (11) 79%	EF5668 (12) 86%	DBL5 (13) 93%	DBL6A (14) 100%
≥#2 PspA for each challenge strain	R36A (12) 86%	BG9739 (12) 100%				
Max score (+) type score	R36A (9) 64%	WU2 (11) 79%	BG9739 (13) 92%	DBL5 (14) 100%		
Max Increase in Days alive	R36A (9) 64%	WU2 (11) 79%	BG9739 (13) 92%	DBL5 (14) 100%		
% protected	R36A (7) 50%	WU2 (10) 64%	DBL5 (11) 79%	EF5668 (12) 86%	DBL6A (13) 92%	EF3296 (14) 100%
Theoretical mixture based on a few testable assumptions (see below)	R36A (10) 64%	BG9739 (12) 86%	DBL5 (13) 92%	EF3296 (14) 100%		

* This is not a unique combination. See table below.

TABLE 37

Combinations where all Challenge Strains have a Vaccine strain with a score of ≥#2				
Number of PspAs in Combination	Combination	Number of #1 strains	Total #1s	Total #1s and #2s
2	R36A +BG9739	8	10	20
3	R36A + BG9739 +WU2	11	15	25
3	R36A +WU2 + DBL5	11	15	21
3	R36A + WU2 + EF5668	11	15	23
3	R36A + WU2 + DBL5	11	15	22

TABLE 38

Pooled Data for Protection against D39 by various PspAs; Days alive for each mouse

Exp.	Log CFU D39	Mice	Days to Death/ immunogen				
			Rx1/R36A D39	JD908 (WU2)	EF5668	All Immune	control
143	4.5	CBA/N			1,1,2,2,2		1,1,2,2,3
E145	4.0	CBA/N	2,3,3,3,4				1, 1, 2, 3, 4
E028 BCG	5.93	BALB/c	3, 3x >21				2,2,2,4
E143	3.0	CBA/N			2,6,3x>10		3,3,3,5,5
E140 BC100	2.81	CBA/N	4,4,5,7,15				2,2,2
E169	2.7	CBA/N	2, 4x >21	2,5,3x >21			1,2,2,2,3
E154	2.6	CBA/N	2,2,3,2x >21				4x 2, 5, >21
All ≤3.0			2,3,3,3,4,4,4 ,5,7,15		1,1,2,2,2		4x 1, 6x 2, 3,3,4
All			4x 2, 5x 3, 3x 4, 5, 7, 15, 9x >21	2,5,3x >21	1,1,2,2,2,2,6 3x >21	1,1,9x 2, 5x 3, 3x 4, 5,5,6,7,15, 15x >21	5x 1, 16x 2, 6x 3, 4, 4, 5,5,5,>21

TABLE 39

Pooled Data for Protection against D39 by various PspAs
Median Days Alive & alive : dead
with corresponding *P* values.

Exp.	Log CFU	Mice	Rx1/R36A D39		JD908 (WU2)		EF5668		All Immune		Control	
	D39		med	a:d	med	a:d	med	a:d	med	a:d	med	a:d
143	4.5	CBA/N					2 n.s.	0:5			2	0:5
E145	4.0	CBA/N	3 n.s.	0:5							2	0:5
E028 BCG	5.93	BALB/c	>21 .029	3:1 n.s.							2	0:4
E143	3.0	CBA/N					>21 n.s.	3:2 n.s.			3	0:5
E140 BC100	2.81	CBA/N	5 0.018	0:5							2	0:3
E169	2.7	CBA/N	>21 .016	4:1 .024	>21 .016	3:2 n.s.					2	0:5
E154	2.6	CBA/N	3 n.s.	2:3 n.s.							2	1:5
All ≤3.0			4 .0008	0:10			2 n.s.	0:5			2	0:13
All			4.5 .0057	9:15 .001 ++	>21 .006	3:2 .0045 +++	4 (2.6) n.s.	3:7 .034 +	5 .0001	15:24 .0002 ++	2	1:32
% alive				38		60		30		38		3
				36		59		28		36		
			Rx1/D39		WU2		EF5668		All immune		controls	

TABLE 40

Pooled Data for Protection against WU2. by various PspAs

Exp.	CFU WU2	Mice	Days to Death/ immunogen									
			FL-R36A	Rx1 BC100	JD108 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 bc100	DBL5 bc100	JS3020 (DBL6A)	control
Dr. Ed, expt.											+++	
lots of prior expts.			+++									
E012	~3.0	CBA/N	15x >21									1,1, 11x 2, 7x 3, 4
E028	6.01	BALB/c	4x >21 0.05/n.s.									4, 6, 6, >21
E084	3.75[1]	CBA/N				3x >15						1,2,2,2,3,3, >15
E125 bc100	3.57	CBA/N					4x >21		4x >21	4x >21		2,2,3,3,3, >21
E129	3.18	CBA/N				5x >23						2,2,2,2,3
E140 BC100	3.43	CBA/N		4x >21								1, 5x 2, 3, 4
E143	3.0	CBA/N						8x >10				1,1,2,2,2,3
E144	3.9	CBA/N									5x >21	5x 2
E172	3.98	CBA/N			5x >21							5x 3
All			19x >21	4x >21	5x >21	8x >21	4x >21	8x >21	4x >21	4x >21	5x >21	6x 1, 33 x 2, 20x 3, 4,4,4,6,6, >21
All Immune			61x >21									

TABLE 41

Pooled Data for Protection against WU2 by various PspAs

Exp.	CFU WU2	Mice	Median days Alive Alive : Dead P value based on Alive : Dead P value calculated compared to pooled controls (in this case 65 control mice) Score									
			FL-R36A	Rx1 BC100	JD108 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 bc100	DBL5 bc100	JS3020 (DBL6A)	control
Dr. Ed, expt.											+++	
lots of prior expts.			+++									
E012	~3.0	CBA/N	>21 15 : 0									1,1, 11x 2, 7x 3, 4
E028	6.01	BALB/c	4x >21									4, 6, 6, >21
E084	3.75[1]	CBA/N				3x >15						1,2,2,2,3,3, >15
E125 bc100	3.57	CBA/N					4x >21		4x >21	4x >21		2,2,3,3,3, >21
E129	3.18	CBA/N				5x >23						2,2,2,2,3
E140 BC100	3.43	CBA/N		4x >21								1, 5x 2, 3, 4
E143	3.0	CBA/N						8x >10				1,1,2,2,2,3
E144	3.9	CBA/N									5x >21	5x 2
E172	3.98	CBA/N			5x >21							5x 3
All			>21 19 : 0 <.0001 +++	>21 4 : 0 <.0001 +++	>21 5 : 0 <.0001 +++	>21 8 : 0 <.0001 +++	>21 4 : 0 <.0001 +++	>21 8 : 0 <.0001 +++	>21 4 : 0 <.0001 +++	>21 4 : 0 <.0001 +++	>21 4 : 0 <.0001 +++	2 1 : 64
% alive			100	100	100	100	100	100	100	100	100	2
			FL-R36A	Rx1 BC100	JD108 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 bc100	DBL5 bc100	JS3020 (DBL6A)	control

WU2 Challenge	days of death	median days of death	alive : dead	P value based on days to death	P value based on alive : dead	Score	% alive	% prot.
All immune	61x >21	>21	61 : 0	<.0001	<.0001	+++	100	100
All controls	6x 1, 33 x 2, 20x 3, 4,4,4,6,6, >21	2	1 : 64				2	2

TABLE 42

Pooled Data for Protection against A66. by various PspAs

Exp.	CFU A66	Mice	Days to Death/ immunogen											
			FL-R36A/ D39	Rx1 BC100	JD908 (WU2)	JS1020 (BG9739)	BG9739 bc100	EF5668	L81905 FL	L81905 bc100	JS5010.3 FL (DBL5)	DBL5 bc100	JS3020 (DBL6A)	control
E169	2.60	CBA/N	5x >21		5x >21									1,1,2,2,6
E152 bc100	2.78	CBA/N					4x >21			4x >21		4x >21		3x 2, 3, 6, 6, >21
E104	3.0	CBA/N				2,8,3x >22					3,4,4,2x >22		2,4,4,5,>22	2,2,2,2,3
E143	3.0	CBA/N						4, 4x >10						2,2,3,3
E140	3.43	CBA/N		4x >21										1,1,1
E172	3.94	CBA/N							5x >21					
E145	3.97	CBA/N	13, 4x >21											1, 2, 2, 2, 4
E121	4.16	CBA/N	3x 3, 2x 4, 5x >21											1, 8x 2, >21
All			3x 3, 2x 4, 13, 14x >21	4x >21	5x >21	2,8,3x >21	4x >21	4, 4x >21	5x >21	4x >21	3,4,4,2x >21	4x >21	2,4,4,5,>21	7x 1, 22x 2, 3x 3, 4, 3x 6, 2x >21
median; A : D			>21 14 : 6	>21 4 : 0	>21 5 : 0	>21 3 : 2	>21 5 : 0	>21 4 : 1	>21 5 : 0	>21 4 : 0	4 2 : 3	>21 4 : 0	4 1 : 4	2 2 : 36
P values			<0.0001 <0.0001	0.0002 0.0001	<0.0001 <0.0001	0.004 0.0075	0.0002 <0.0001	0.0006 0.006	<0.0001 <0.0001	0.0002 0.0001	0.0025 n.s.	0.0002 0.0001	0.015 n.s.	
Mini Pools			R36A/Rx1/WG44.1		JD908	BG9739		EF5668	L81905		DBL5 3, 4, 4, 4, 6x >21		DBL6A	Control
			>21 18 : 6		>21 5 : 0	>21 8 : 2		>21 4 : 1	>21 9 : 0		>21 6 : 4		4 1 : 4	2 2 : 36
P valaues rank/a:d			<0.0001		<0.0001 <0.0001	<0.0001		0.0006 0.006	<0.0001		0.0004		0.015 n.s.	
Score			+++		+++	+++		+++	+++		+++		+±	
% alive			72		100	80		75	100		60		20	5
			71		100	79		74	100		58		16	0
A66 challenge			R36A/Rx1/WG44.1		JD908	BG9739		EF5668	L81905		DBL5		DBL6A	

A66 challenge	days of death	median days alive	alive : dead	P - days to death	P - alive : dead	Score	% alive	% protected
All immune	2, 2, 4x 3, 7x 4, 5, 8, 13, 50x >21	>21	50 : 16	<0.0001	<0.0001	+++	76	75
All controls	7x 1, 22x 2, 3x 3, 4, 3x 6, 2x >21	2	2 : 36				5	0

TABLE 43

Pooled Data for Protection against EF10197. by various PspAs

Exp.	CFU EF 10197	Mice	Days to Death/ immunogen						
			Rx1 BC100	JS1020 (BG9739)	L81905	JS3020 (DBL6A)	EF5668	JS5010.3 FL (DBL5)0	control
E140	3.00	CBA/N	5x >21						2,2,2
MI BCG	2.70	CBA/N	*						2,2,2,2,2
E129	3.34	CBA/N		8, 4x >23					2,2,2,2,9

* This was a passive protection study. Its controls have been included to increase the numbers of control mice.

TABLE 44

Pool of Pools for protection agaisnt EF10197					
line	Gr up Descripti n	Delay in death and/ r survival		Survival	
		days to death (medain)	*P* values etc.	alive : dead	*P* values etc.
1a	Rx1 (E140)	5x >21	0.017 vs 1b 0.0013 vs 4b	5 : 0	0.018 vs 1b 0.008 vs 4b
3a	JS1020 (E129)	8, 4x >23	0.0007 vs 3b	4 : 1	0.024 vs 3b
4a	all immune	8, 9x >21	<0.0001 vs 4b	9 : 1	0.0002 vs 4b
1b	Rx1 controls (E140)	2,2,2		0 : 3	
2b	MI BCG	2,2,2,2,2		0 : 5	
3b	JS1020 cont. (E129)	2,2,2,2,9		0 : 5	
4b	all controls (without MI BCG)	2,2,2,2,2,2,2,9		0 : 8	

TABLE 45

Summary of protection against EF10197							
Immunogen	alive : dead	% alive	% protected	median DOD	*P* time alive	*P* alive : dead	Score*
Rx1	5 : 0	100	100	>21	0.017	0.018	+++
JS1020	4 : 1	80	80	>21	0.0007	0.024	+++
all immune	9 : 1	90	90	>21	<0.0001	0.0002	+++
all controls	0 : 8	0	0	2	--	--	--

* +++ = statistically significant protection against death with ≥50% protected.

TABLE 46

Pooled Data for Protection against ATCC6303. by various PspAs								
Exp.	CFU ATCC 6303	Mice	Days to Death/ immunogen					
			Rx1 BC100	JS1020 (BG9739)	L81905	JS3020 (DBL6A)	EF5668	JS5010.3 FL (DBL5)0	control
E140	2.30	CBA/N	5x >21						4, 4x 5
E129	3.80	CBA/N		n.v.					

TABLE47

Pool of Pools for protection agaisnt ATCC6303						
line	Group Description	Delay in death and/or survival			Survival	
		days to death	(medain)	*P* values etc.	alive : dead	*P* values etc.
1a	Rx1 (E140)	5x >21	(>21)	0.0040	5 : 0	0.004
1b	RX1 controls (E140)	4, 4x 5	5	--	0 : 5	--

TABLE 48

Summary of protection against ATCC6303							
Immunogen	alive : dead	% alive	% protected	median DOD	*P* time alive	*P* alive : dead	Score*
Rx1	5 : 0	100	100	>21	0.004	0.004	+++
Rx1 controls	0 : 5	0	0	5	--	--	--

* +++ = statistically significant protection against death with ≥50% protected.

TABLE 49

Pooled Data for Protection against BG9739. by various FL PspAs

Exp.	CFU BG9739	Mice	Days to Death/ immunogen											
			R36A FL	BC100 (Rx1)	JD908 (WU2)	JS1020 (BG9739)	bc100 (BG9739)	EF3296 FL	EF5668 FL	bc100 (L81905)	JS5010.3 FL (DBL5)	bc100 (DBL5)	JS3020 (DBL6A)	control
E140	2.76	CBA/N		3,3,10,11										2,2,3
E104	2.89	Xid				6,6,7,8,8					2,2,2,3,4		2,2,2,2,3	2,2,3,5,5
E125	3.56	CBA/N					5,5,5,7			4,5,13, >21		2,2,2,4		3,3,4,4,5,6
E172	3.71	CBA/N			6, 7, 3x >21									3,4,6,6,7
E124	3.76	Xid									2,2,2,2,3		2,2,2,2,9	2,2,2,2,2
E084	4.05	BALB/c				4x2, 2x >14								9x2
E144	4.09	Xid	2,3,3,6,>21					2,3,3,7, >10	2,3,3,3,4					2,2,2,3,3
All			2,3,3,6,>21	3,3,10,11	6,7,3x >21	4x2, 6, 6, 7, 8, 8, 2x >21	5,5,5,7	2,3,3,7 >21	2,3,3,3,4		7x 2, 3, 3, 4		8x 2, 3, 9	21x 2, 7x 3, 3x 4, 3x 5, 3x 6, 7
median			3	3, 10	>21	6	5	3	3	5, 13	2	2	2	2
a : d			1 : 4	0 : 4	3 : 2	2 : 9	0 : 4	1 : 4	0 : 5	1 : 3	0 : 10	0 : 4	0 : 10	0 : 38
P rank														
P a:d														

TABLE 50

Pooled Data for Protection against BG9739. by bc100s and FL PspAs

Exp.	CFU BG9739	Mice	Days to Death/ immunogen											
			R36A FL	BC100 (Rx1)	JD908 (WU2)	JS1020 (BG9739)	bc100 (BG9739)	EF3296 FL	EF5668 FL	bc100 (L81905)	JS5010.3 FL (DBL5)	bc100 (DBL5)	JS3020 (DBL6A)	control
E140	2.76	CBA/N		3,3,10,11										2,2,3
E104	2.89	Xid				6,6,7,8,8					2,2,2,3,4		2,2,2,2,3	2,2,3,5,5
E125	3.56	CBA/N					5,5,5,7			4,5,13, >21		2,2,2,4		3,3,4,4,5,6
E172	3.71	CBA/N			6, 7, 3x >21									3,4,6,6,7
E124	3.76	Xid									2,2,2,2,3		2,2,2,2,9	2,2,2,2,2
E084	4.05	BALB/c				4x2, 2x >14								9x2
E144	4.09	Xid	2,3,3,6, >21					2,3,3,7, >10	2,3,3,3,4					2,2,2,3,3
FL + bc100 BG9739			R36A/Rx1/D39		WU2	BG9739		EF3296	EF5668	L81905	DBL5		DBL6A	Cont.
All			2, 4x 3, 6, 10, 11, >21		6, 7, 3x >21	4x 2, 3x 5, 2x 6, 2x 7, 2x 8, 2x >21		2,3,3,7, >21	2,3x 3,4	4,5,13, >21	10x 2, 3, 3, 4, 4		8x 2, 3, 9	21x 2, 7x 3, 3x 4, 3x 5, 3x 6, 7
median days alive			3		>21	6		3	3	5, 13	2		2	2
alive : dead			1 : 8		3 : 2	2 : 13		1 : 4	0 : 5	1 : 3	0 : 14		0 : 10	0 : 38
P - days alive			0.0096		<0.0001	0.0013		n.s.	n.s.	0.0022	n.s.		n.s.	
P- alive : dead			n.s.		0.0008	n.s.		n.s.	n.s.	n.s.	n.s.		n.s.	
Score			+		+++	+		0+	0	+±	0		0	
% alive			11		60	13		25	0	25	0		0	0
% protected			11		60	13		25	0	25	0		0	0
BG9739 challenge			R36A/Rx1/D39		WU2	BG9739		EF3296	EF5668	L81905	DBL5		DBL6A	Cont.

BG9739	days of death	median days of death	alive : dead	P value based on days to death	P value based on alive : dead	Score	% Alive	%	
All immune		3	8 : 59	0.009	0.023	++	12	12	
All controls		2	0 : 38						

TABLE 51

Pooled Data for Protection against EF3296. by various PspAs

Exp.	CFU EF3296	Mice	Days to Death/ immunogen					
			Rx1 BC100	JD908 WU2	JS1020 (BG9739)	JS5010.3 FL (DBL5)	JS3020 (DBL6A)	control
E84[1]	3.99	BALB/c			4x 2, >14			9x 2
E140	2.92	CBA/N	3, 4, 6, >21					3,3,3
E104	3.11	CBA/N			4,5,5,5,6	2,2,2,3,3	2,2,3,4,5	2,2,2,3,4
E124	3.94	CBA/N				1,1,2,2,2	1,1,2,2,2	1,1,2,2,2
E172	4.06	CBA/N						3, 4x 6
All			3, 4, 6, >21	3,3,5,5,>21	4x 2, 4, 3x 5, 6, >21	1,1,5x 2, 3,3	1, 1,5x 2, 3, 4, 5	1, 1, 15x 2, 5x 3 4, 4x 6
median days to death			5	5	4.5	2	2	2
alive : dead			1 : 3	1 : 4	1 : 9	0 : 9	0 : 10	0 : 27
P - days to death			0.0077	0.0094	n.s.	n.s.	n.s.	
P - alive : dead			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Score			+±	+±	0+	0	0	
% alive			25	20	10	0	0	0
% prot.			25	20	10	0	0	0
Best								
EF3296 challenge			Rx1 BC100	JD908 WU2	JS1020 (BG9739)	JS5010.3 FL (DBL5)	JS3020 (DBL6A)	control

EF3296 challenge	median days alive	alive : dead	P - days to death	P - alive : dead	Score	% alive	% prot
All immune	3	3 : 35	n.s.	n.s.	0	8	8
All control	2	0 : 27					

TABLE 52

Pooled Data for Protection against EF5668. by various FL-PspAs and bc100s

Exp.	CFU EF 5668	Mice	Days to Death/ immunogen								
			R36A	Rx1 BC100	JD908 (WU2)	JS1020 (BG9739)	EF3296	EF5668	L81905	JS5010.3 FL DBL5	JS3020 DBL6A	control
E143	3.0	CBA/N						5x >10				1,1,2,2, >10
E140	3.59	CBA/N		4,6,12,>21								2,4,6
E171	3.69	CBA/N			2, 2, 2, 3, >21				3,3,4, 2x >21			1,3,6,6,7
E124	3.90	CBA/N								3,3,3x >15	3,4,5,6,6	3,3,3,4,9
E145	3.94	CBA/N	3, 4, 4, 16, >19			2, 10, 3x >19	2, 4, 13, 2x >19					2, 3, 3, 4, >21
Pool			3, 3x 4, 6, 12, 16, 2x >21		2, 2, 2, 3, >21	2, 10, 3x >21	2, 4, 13, 2x >21	5x >21	3,3,4, 2x >21	3, 3, 3x >21	3,4,5,6,6	3x 1, 4x 2, 6x 3, 3x 4, 3x 6, 7, 9, 2x >21
median days alive			6		2	>21	13	>21	4	>21	5	3
alive : dead			2 : 7		1 : 4	3 : 2	2 : 3	5 : 0	2 : 3	3 : 2	0 : 5	2 : 21
P - days alive			0.013		n.s.	0.0187	n.s.	0.001	n.s.	n.s.	n.s.	
P - alive : dead			n.s.		n.s.	0.027	n.s.	0.0002	n.s.	0.027	n.s.	
Score			+		0	+++	0+	+++	0+	+	0+	
% alive			22		25	60	40	100	40	60	0	9
% prot			14		18	56	34	100	34	56	-10	9
EF5668			R36A/Rx1/D39		WU2	BG9739	EF3296	EF5668	L81905	DBL5	DBL6A	control

Summary of protection against EF6796						
Immunogen	alive : dead	% alive	% protected	median DOD	*P* -time alive	*P* alive vs dead
Rx1	4 : 0	100	100	>21	0.029	0.029
controls	0 : 3	0	0	1	--	--

* +++ = statistically significant protection from death with ≥ 50% protected;

TABLE 53

Pooled Data for Protection against DBL6A. by various FL PspAs and bc100 PspAs

Exp.	CFU DBL6A	Mice	Days to Death/ immunogen											
			BC100 Rx1	R36A	JD908 WU2	JS1020 BG9739	bc100 BG9739	EF3296	EF5668	L81905 FL	bc100 L81905	JS5010.3 DBL5	bc100 DBL5	JS3020 DBL6A	control
E171	2.69	CBA/N			6,7,8,9, >21					3,3,7,9, >21					2,3,4,6,6
E152	3.24	CBA/N					15, 3x >21				7,16, 2x >21		8, 10, 13, 21		3x 3, 4, 3x 6
E140	3.25	CBA/N	4x >21												4, 7, 7
E146	3.57	CBA/N		7, 8, 10, 2x >21				6, 8, 9, 10, 10	10, 13, 3x >21			7,8,12, 13,13		9, 4x >21	4,4,5,5,18
E129	4.14	CBA/N				3,6,8,10, 13									4,5,6,8,>23
Total															
Name of Pools			R36A/Rx1/D39		WU2	BG9739		EF3296	EF5668	L81905		DBL5		DBL6A	controls
Pooled data			7, 8, 10, 6x >21		6,8,9, >21	3,6,8,10,13,15, 3x >21		6, 8, 9, 10, 10	10, 13, 3x >21	3,3,7,7,9,16, 2x >21		7,8,8,10,12, 3x 13, 21		9, 4x >21	2, 4x 3, 6x 4, 3x 5, 6x 6, 7, 7, 8, 18, >21
median days alive			>21		8.5	13		9	>21	8		12		>21	5
alive : dead			6 : 3		1 : 3	3 : 6		0 : 5	3 : 2	2 : 6		0 : 9		4 : 1	1 :24
P - days alive			<0.0001		0.0082	0.0025		0.0036	0.0001	0.037		0.002		<0.0001	
P - alive : dead			0.0019		n.s.	0.048		n.s.	0.0093	n.s.		n.s.		0.0009	
Score			+++		+±	++		+±	+++	+±		+±		+++	
			67		25	33		0	60	25		0		80	4
			66		22	30		-4	58	22		-4		79	0
DBL6A challenge			R36A/Rx1/D39		WU2	BG9739		EF3296	EF5668	L81905		DBL5		DBL6A	controls

DBL6A challenge	days of death	median days of death	alive : dead	*P* value based on days to death	*P* value based on alive : dead	Score	% alive	% prot.
All immune		12.5	19 : 35	<0.0001	0.0019	++	35	33
All control		5	1 : 24					

TABLE 54

Pooled Data for Protection against BG9163 by various PspAs

Exp.	CFU BG9163	Mice	Days to Death/ immunogen				
			Rx1	Rx1.BCG	JS1020 (BG9739)	all immune	control
E169	2.67	CBA/N	5x >24				4,5,8,8,>24
E140	3.14	CBA/N	n.v.				
E129	4.0	CBA/N			12, 4x >23		7, 9,9,13, >23
E028	6.217	CBA/N		6, 3x >21			5,6,8,10
Immunogens			Rx1/R36A/D39		BG9739	all immune	control
Pooled Data			6, 8x >21		12, 4x >21	6, 12, 12x >21	4,5,7,8,8,9, 9,12,2x >21
median days alive			>21		>21	>21	8.5
alive : dead			8 : 1		4 : 1	12 : 2	2 : 8
P - days alive			0.0086		0.0097	0.0027	
P - alive : dead			0.0045		0.047	0.0022	
% alive			89		80	86	20
% prot.			86		75	83	0
score			+++		+++	+++	
BG9163 Challenge			Rx1/R36A/D39		BG9739	all immune	control

EF5668	days of death	median days of death	alive : dead	*P* value based on days to death	*P* value based on alive : dead	Score	% alive	% prot.
All immune		8	18:26	0.0015	0.005	++	41	35
All control		3	2:21					

TABLE 55

Pooled Data for Protection against L81905. by various FL-PspAs

Exp.	CFU L81905	Mice	Days to Death/ immunogen											
			R36A	BC100 (Rx1)	JD908 (WU2)	JS1020 (BG9739)	bc100 BG9739	EF3296	EF5668	bc100 L81905	JS5010.3 (DBL5)	bc100 (DBL5)	JS3020 (DBL6A)	control
E172	2.45	CBA/N			3,4,5,6,6									3,3,4,4,4
E140	3.11	CBA/N		2,5,5,6,8										2,2,2,3,3
E084	3.86	BALB				2, 2, 5x >14								1, 8x 2
E104	-3.5	CBA/N				3,7,8,8,11					3,3,3,2x >22		3,4,5,5,6	2, 4, 4, 4, 5
E124	-3.5	CBA/N									2, 2, 2, 2, 3		2,2,2,3,5	1,2,2,2,2
E125	3.6	CBA/N					5,6,8,8			3,4,6,8		4,5,5,5		2,2,3,5,5,5
E144	4.11	CBA/N	3,3,5,6, >10					6,6,6, 2x >10	2,2,3,3,3					2, 2, 3x 3
All			3,3,5,6, >21		3,4,5,6,6	2,2,3,7,8,8, 11, 5x >21	5,6,8,8	6,6,6, 2x >10	2,2,3,3,3	3,4,6,8	4x 2, 4x 3, 2x >21	4,5,5,5	3x 2, 3, 3, 4, 3x 5, 6	1, 1, 20x 2 8x 3. 6x 4. 4x 5
median			5	5	5	>21	7	6	3	5	3	5	3.5	2
alive: dead			1:4	0:5	0:5	5:7	0:4	2:3	0:5	0:4	2:8	0:4	0:10	0:40
P rank														
P a:d														
score														

TABLE 56

Protection against L81905. by various

bc100s & FL-PspAs pooled together

Exp.	CFU L81905	Mice	Days to Death/ immunogen											
			R36A	BC100 (Rx1)	JD908 (WU2)	JS1020 (BG9739)	bc100 BG9739	EF3296	EF5668	bc100 L81905	JS5010.3 (DBL5)	bc100 (DBL5)	JS3020 (DBL6A)	control
E172	2.45	CBA/N			3,4,5,6,6									3,3,4,4,4
E140	3.11	CBA/N		2,5,5,6,8										2,2,2,3,3
E084	3.86	BALB				2, 2, 5x >14								1, 8x 2
E104	-3.5	CBA/N				3,7,8,8,11					3,3,3,2x >22		3,4,5,5,6	2, 4, 4, 4, 5
E124	-3.5	CBA/N									2, 2, 2, 2, 3		2,2,2,3,5	1,2,2,2,2
E125	3.6	CBA/N					5,6,8,8			3,4,6,8		4,5,5,5		2,2,3,5,5,5
E144	4.11	CBA/N	3,3,5,6, >10					6,6,6, 2x >10	2,2,3,3,3					2, 2, 3x 3
Pooled			2,3,3, 3x 5, 6,6,8,>21		3,4,5,6,6	2,2,3,5,6,7,4x 8,11, 5x >21		6,6,6, 2x >10	2,2,3,3,3	3,4,6,8	4x 2, 4x 3, 4,5,5,5, 2x >21		3x 2, 3, 3, 4, 3x 5, 6	1, 1, 20x 2 8x 3. 6x 4. 4x 5
median days alive			5		5	8		6	3	5	3		3.5	2
alive: dead			1:9		0:5	5:11		2:3	0:5	0:4	2:12		0:10	0:40
P - days alive			0.0005		0.0035	<0.0001		0.0002	n.s.	0.01	0.035		0.044	
P - alive : dead			n.s.		n.s.	0.0001		0.01	n.s.	n.s.	n.s.		n.s.	
score			+		+	++		++	0	+	+		+	
% alive			10		0	31		40	0	0	14		0	0
% protected														
challenge with L81905			R36A/Rx1/D39		WU2	BG9739		EF3296	EF5668	L81905	DBL5		DBL6A	controle

L81905 challenge	days of death	median days of death	alive : dead	*P* value based on days to death	*P* value based on alive : dead	Score	% alive	% prot.
All immune		5	10 : 59	<0.0001	0.008	++	14	14
All control		2	0 : 40					

TABLE 57

Pooled Data for Pr tection against DBL5 by various FL-PspAs & bc100s

Exp.	CFU DBL5	Mice	Days to Death/ immunogen									
			R36A	BC100 Rx1	JS1020 BG9739	bc100 JS1020	EF5668	bc100 L81905	JS5010.3 DBL5	bc100 DBL5	JS3020 DBL6A	control
E84[1]	3.90	BALB/c			6x 2							9x 2
E140	3.27	CBA/N		4,4,5,5, 5								2,2,2
E104	3.39	Xid			3,3,6, >22, >22				7,7,15, >22, >22		2,2,4,5,5	2,4x 3
E124	3.76	Xid							2,2,2,5,> 15		5x 2	1,1,2,2,2
E125	3.81	CBA/N				3,3,4,5		3,3,4,4		2,2,2,>21		5x 2, 5
E144	4.13	XID	3,3,3,3, >10				2,2,3,4, 4					5x 2
total												
name of pool			R36A/Rx1/D39		BG9739		EF5668	L81905	DBL5		DBL6A	controls
pooled data			4x 3, 2x 4, 3x 5, >21		6x 2, 4x 3, 4, 5, >21, >21		2,2,3,4,4	3,3,4,4	6x 2, 5, 7, 7, 15, 4x >21		7x 2, 4, 5 5	1, 1, 26x 2, 4x 3, 5
median days alive			4		3		3	3.5	6		2	2
alive : dead			1 : 9		2 : 12		0 : 4	0 : 4	4 : 10		0 : 10	0 : 32
P - days alive			<0.0001		0.0063		.041	0.001	0.0025		n.s.	
P - alive : dead			n.s.		n.s.		n.s.	n.s.	0.0056		n.s.	
Score			+		+		+*	+	++		0	
% alive			10		14		0	0	29		0	0
% protected			10		14		0	0	29		0	0
DBL5 challenge			R36A/Rx1/D39		BG9739		EF5668	L81905	DBL5		DBL6A	controls

[1] This immunization was with cell eluted PspA. Note BALB/cJ mice were used. Also note 10^4 Challenge CFU.

DBL5 challenge	days of death	median days of death	alive : dead	*P* value based on days to death	*P* value based on alive : dead	Score	% alive	% prot.
All immune		3.5	7 : 49	<0.0001	0.034	++	3.6	3.6
All control		2	0 : 33					

TABLE 58

Pooled Data for Protection against EF6796 by various PspAs

Exp.	CFU WU2	Mice	Days to Death/ immunogen						
			Rx1 BC100	JS1020 (BG9739)	L81905	JS3020 (DBL6A)	JS5010.3 FL (DBL5)	DBL5 bc100	control
E140	3.75	CBA/N	4x >21						1,1,1
E28	?	BALB	n.v.						

TABLE 59

Pool of Pools for protection agaisnt EF6796

line	Group Description	Delay in time to death and/or survival			Pr tecti n against death	
		days to death (medain DOD)		*P* values etc.	alive : dead	*P* values etc.
1a	Rx1	4x >21	(>21)	0.029	4 : 0	0.029
1b	Rx1 controls	1,1,1	(1)	--	0 : 3	--

TABLE 60

Pooled Data for Protection against BG7322. by various FL- PspAs and bc100s

Exp.	CFU BG 7322	Mice	Days t Death/ immunogen										
			D39/ R36A	Rx1 BC100	JD908 (WU2)	bc100 BG9739	EF3296	EF5668	bv100 L81905	JS5010.3 DBL5	bc100 DBL5	JS3020 DBL6A	control
E171	2.78	CBA/N			10, 15, 3x >21								1, 3, 6, 6, 7
S143	3.0	CBA/N						7, 8x >10					2,2,4,5,7,7, 8,8
E140 BC100	3.14	CBA/N		4x >21									3, 6, 6, >21
E152	3.11	CBA/N				12, 13, 16, >21			10, 12, 13, >21		>21, >21, >21, >21		6, 7, 7, 8, 8, 9, 14
E146	3.57	CBA/N	18, 20, 3x >21				5, 3x 6, 10			6,10,11, 11,19		4, 8, 11, 18, >21	4, 5, 5, 6, >21
E169	3.94	CBA/N	5x >21										2,5,5,6,7
Immunogens			R36A/Rx1/D39		JD908	BG9739	EF3296	EF5668	L81905	DBL5		DBL6A	Cont.
Pools			18, 20, 12x >21		10, 15, 3x >21	12, 13, 16, >21	5, 3x 6, 10	7, 8x >21	10, 12, 13, >21	6,10,11, 11,19 >21, >21, >21, >21		4, 8, 11, 18, >21	1, 3x 2, 3,3, 4, 4, 5x 5, 7x 6, 6x 7, 4x 8, 9, 14, 2x >21
median day alive			>21		>21	14.5	6	>21	12.5	>21		11	6
alive : dead			9:0		3:2	1:3	0:5	8:1	1:3	4:5		1:3	2:32
P - days alive			<0.0001		0.0007	0.001	n.s.	<0.0001	0.0013	0.0002		0.028	
P - alive : dead			<0.0001		0.004	n.s.	n.s.	<0.0001	n.s.	0.0076		n.s.	
% alive			100		60	25	0	89	25	80		25	6
% protected			100		57	22	0	88	22	79		22	6
Score			+++		+++	+±	0	+++	+±	+++		+±	
BG7322 Challenge			R36A/Rx1/D39		JD908	BG9739	EF3296	EF5668	L81905	DBL5		DBL6A	Cont.

BG7322 Challenge	median days of death	alive : dead	*P* value based on days to death	*P* value based on alive : dead	Score	% alive	% prot.
All immune	>21	30 : 25	<0.0001	<0.0001	+++	55	52
All controls	6	2 : 32					

WHAT IS CLAIMED IS

1. An isolated amino acid molecule consisting of residues 1 to 115, 1 to 260, 192 to 588, 192 to 299, or residues 192 to 260 of pneumococcal surface protein A of *Streptococcus pneumoniae*.

2. An isolated DNA molecule consisting of a fragment of a pneumococcal surface protein A gene of *Streptococcus pneumoniae* encoding the isolated amino acid molecule of claim 1.

3. A PCR primer consisting essentially of the isolated DNA molecule of claim 2.

4. A hybridization probe consisting essentially of the isolated DNA molecule of claim 2.

5. An immunological composition comprising the amino acid molecule of claim 1.

6. An isolated DNA molecule consisting of nucleotides 1 to 26, 1967 to 1990, 161 to 187, 1093 to 1117 or 1312 to 1331, or 1333 to 1355 of a pneumococcal surface protein A gene of *Streptococcus pneumoniae*.

7. A PCR primer consisting essentially of the isolated DNA molecule of claim 6.

8. A hybridization probe consisting essentially of the isolated DNA molecule of claim 6.

9. An isolated DNA molecule consisting of a fragment of a pneumococcal surface protein A gene of *Streptococcus*

pneumoniae consisting of a nucleotide sequence (5′ to 3′) selected from

CCGGATCCAGCTCCTGCACCAAAAAC;
GCGCGTCGACGGCTTAAACCCATTCACCATTGG;
CCGGATCCTGAGCCAGAGCAGTTGGCTG;
CCGGATCCGCTCAAAGAGATTGATGAGTCTG;
GCGGATCCCGTAGCCAGTCAGTCTAAAGCTG;
CTGAGTCGACTGGAGTTTCTGGAGCTGGAGC;
CCGGATCCAGCTCCAGCTCCAGAAACTCCAG;
GCGGATCCTTGACCAATATTTACGGAGGAGGC;
GTTTTTGGTGCAGGAGCTGG;
GCTATGGCTACAGGTTG;
CCACCTGTAGCCATAGC;
CCGGATCCAGCGTGCCTATCTTAGGGGCTGGTT; and
GCAAGCTTATGATATAGAAATTTGTAAC.

10. A PCR primer consisting essentially of at least one isolated DNA molecule of claim 9.

11. A hybridization probe consisting essentially of at least one isolated DNA molecule of claim 9.

12. PCR probe(s) which distinguishes between *pspA* and *pspA*-like nucleotide sequences.

13. PCR probe(s) which hybridizes to both *pspA* and *pspA*-like nucleotide sequences.

14. A PspA extract prepared by a process comprising

growing pneumococci in a first medium containing choline chloride,

eluting live pneumococci with a choline chloride containing salt solution, and

growing the pneumococci in a second medium containing an alkanolamine and substantially no choline.

15. A PspA extract prepared by

growing pneumococci in a first medium containing choline chloride,

eluting live pneumococci with a choline chloride containing salt solution,

growing the pneumococci in a second medium containing an alkanolamine and substantially no choline, and purifying PspA by isolation on a choline-Sepharose affinity column.

16. An immunological composition comprising the extract of claim 14.

17. An immunological composition comprising the extract of claim 15.

18. An immunological composition comprising full length PspA.

19. A method for enhancing immunogenicity of a PspA-containing immunological composition comprising including in said composition the C-terminal portion of PspA.

20. An immunological composition comprising at least two PspAs.

21. The immunological composition of claim 20 wherein the PspAs are from different groups based on RFLP.

22. PCR amplification product from a primer as claimed in claims 3, 7, 10, 12 or 13.

23. An isolated DNA molecule consisting of a nucleotide sequence homologous to a portion of pspA.

ABSTRACT OF THE DISCLOSURE

PspAs, portions thereof, DNA therefor, and immunological compositions, primers and probes based thereon are disclosed claimed.

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Under 37 CFR § 1.63; includes reference to PCT International Applications)

CURTIS, MORRIS & SAFFORD, P.C.
File No.: 454312-2400

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention ENTITLED: *PNEUMOCOCCAL GENES, PORTIONS THEREOF, EXPRESSION PRODUCTS THEREFROM, AND USES OF SUCH GENES, PORTIONS AND PRODUCTS*, the specification of which _X_ is attached hereto __ was filed on __ as __ United States __ PCT Application No. __ , with amendments through __ (if applicable, give details).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT International application(s) designating at least one country other than the United State of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

Country (or PCT)	Application Number:	Filed (Day/Month/Year)	Priority Claimed: Yes	No

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. (or U.S.-designating PCT) Application(s) [list additional applications on separate page]:

U.S. Serial No.:	Filed (Day/Month/Year)	PCT Application No.	Status (patented, pending, abandoned)
08/226,844			
08/093,907			
07/889,918			
08/482,981			
08/458,399			

DECLARATION FOR PATENT APPLICATION
AND POWER OF ATTORNEY (Under 37 CFR § 1.63)

CMS Docket No. 454312-2400

Prior U.S. (or U.S.-designating PCT) Application(s) [list additional applications on separate page]:

U.S. Serial No.:	Filed (Day/Month/Year)	PCT Application No.	Status (patented, pending, abandoned)
08/446,201			
08/246,636			
08/048,896			
07/835,698			
07/656,773			
08/319,795			
08/072,070			

I hereby appoint William S. Frommer, Reg. No. 25,506, Robert F. Kirchner, Reg. No. 31,034, Thomas J. Kowalski, Reg. No. 32,147, Pamela Salkeld, Registration No. 38,607, and Curtis, Morris & Safford, P.C., Registration No. 12,761, or their duly appointed associate, my attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and specify that all communications about the application are to be directed to the following correspondence address:

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Direct all telephone calls to:
(212) 840-3333
to the attention of:
William S. Frommer

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

INVENTOR(S):

Signature: _____ Date: _____

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Signature: _____ Date: _____

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Signature: _____ Date: _____

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Signature: _____ Date: _____

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Signature: _____ Date: _____

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Signature: _____ Date: _____

Post Office Address(es) of inventors [if different from residence]: